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Dopaminergic Regulation of Secretory Granule–Associated Proteins in Rat Intermediate Pituitary

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Abstract: The biosynthesis of peptides requires the synthesis of the prohormone, several biosynthetic processing enzymes, and other granule constituents. We have investigated the regulated expression of proopiomelanocortin (POMC) and five enzymes essential for the processing of POMC to smaller, bioactive peptides in intermediate pituitary melanotropes. Rats were treated with a dopaminergic agonist (bromocriptine) or antagonist (haloperidol) for periods ranging from 1 h to 5 days, followed by analyses of mRNA levels and protein biosynthetic rates. Multiplex RNase protection assays showed that bromocriptine treatment caused a striking decrease in POMC mRNA levels, and significant decreases in mRNA levels for prohormone convertase 2 (PC2), carboxypeptidase H (CPH), and peptidylglycine α-amidating monooxygenase (PAM). Smaller increases in mRNA levels were seen after haloperidol stimulation. Protein biosynthetic rates changed more profoundly than mRNA levels at short drug treatment times, indicating a role for translational effects after treatment with bromocriptine and with haloperidol. The homogeneous population of melanotropes in the intermediate lobe of the pituitary allows a quantitative analysis of transcript levels and biosynthetic rates. POMC mRNA levels are 200–1,000-fold higher than levels of any of the processing enzyme mRNAs, and POMC biosynthetic rates exceed those of PC2, PC1, and PAM by 1,000–10,000-fold. **Key Words:** Proopiomelanocortin—Prohormone convertase 1—Prohormone convertase 2—Peptidylglycine α-amidating monooxygenase—Cytochrome b_{561}—Translation.
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Biologically active peptides are synthesized from large, inactive precursors by a series of co- and post-translational modifications (Mains and Eipper, 1990; Eipper et al., 1992; Seidah and Chretien, 1992; Seidah et al., 1993; Steiner et al., 1993*a,b*). Proopiomelanocortin (POMC), the common precursor to several peptide hormones, including adrenocorticotropic hormone (ACTH), α-melanotropin (α-MSH), and β-endorphin, is synthesized in large amounts in anterior pituitary corticotropes, intermediate pituitary melanotropes, arcuate nucleus, and nucleus of the solitary tract neurons (Civelli et al., 1982; Khachaturian et al., 1985). POMC processing is cell type specific, presumably reflecting tissue-specific expression of a set of granule-associated enzymes that includes prohormone convertases (PC1 and PC2), carboxypeptidase H (CPH), peptidylglycine α-amidating monooxygenase (PAM), and cytochrome b_{561} ($Cytb_{561}$) (Mains and Eipper, 1990) (Fig. 1).

All of the major endoproteolytic cleavages of POMC occur at pairs of basic amino acids and can be catalyzed by the subtilisin-like endoproteases PC1 and PC2 (Benjannet et al., 1991; Lindberg, 1991; Thomas et al., 1991; Seidah and Chretien, 1992; Bloomquist and Mains, 1993; Zhou et al., 1993). Corticotropes express primarily PC1 mRNA, and melanotropes express high levels of PC2 mRNA along with PC1 mRNA (Seidah et al., 1990; Bloomquist et al., 1991; Hakes et al., 1991; Day et al., 1992). It is interesting that PC1 and PC2 cleave POMC sequentially, with PC1 catalyzing the initial steps of POMC processing and generating ACTH biosynthetic intermediate and β-lipotropin (Fig. 1) (Zhou et al., 1993). These cleavages occur with similar kinetics in the anterior and intermediate lobes of the pituitary. PC2 then catalyzes the later steps in the biosynthetic pathway, which are restricted to melanotropes, generating Lys_0-γ_3-melanotropin and β-endorphin(1–27) (Mains and Eipper, 1990; Zhou et al., 1993). Completion of POMC processing in the pituitary requires removal of Lys and Arg residues

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Abbreviations used: ACTH, adrenocorticotropic hormone; CPH, carboxypeptidase H; CSFM, complete serum-free medium; $Cytb_{561}$, cytochrome b_{561}; DMEM, Dulbecco's modified Eagle medium; α-MSH, α-melanotropin; nt, nucleotides; PAGE, polyacrylamide gel electrophoresis; PAM, peptidylglycine α-amidating monooxygenase; PC1, prohormone convertase 1; PC2, prohormone convertase 2; POMC, proopiomelanocortin; SDS, sodium dodecyl sulfate; SH, solution hybridization.

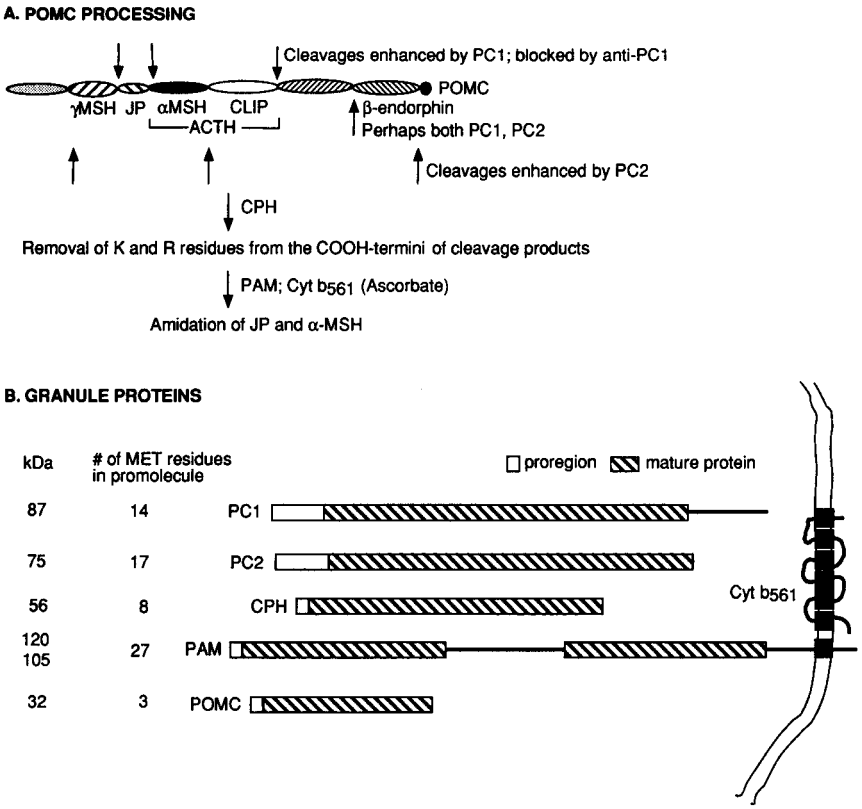


FIG. 1. Schematic diagram of granule-associated proteins involved in POMC processing. **A:** The endoproteolytic cleavages of POMC by PC1 and PC2 are shown by arrows (Mains and Eipper, 1990; Bloomquist et al., 1991; Zhou et al., 1993). Steps catalyzed by CPH and PAM along with $Cytb_{561}$ are indicated. **B:** The lumen of the secretory granule is shown to the left of the lipid bilayer. The topology of $Cytb_{561}$ is shown as determined by Fleming and Kent (1991). The apparent molecular mass (kDa) and the number of methionine residues present in each promolecule are indicated (Fricker et al., 1989; Bloomquist et al., 1991; Srivastava et al., 1994). The granule proteins are drawn to scale. αMSH, α-melanotropin.

from the COOH-termini of the PC1 and PC2 cleavage products by the exopeptidase CPH (Fricker, 1988). After the action of PC1, PC2, and CPH, PAM catalyzes the copper-, ascorbate-, and molecular oxygen–dependent α-amidation of joining peptide and α-MSH (Eipper et al., 1992). The level of ascorbate in the secretory granules is maintained by reduction of the semidehydroascorbate produced during the amidation reaction using electrons imported by the membrane-associated protein $Cytb_{561}$ (Kent and Fleming, 1987).

Because most of the enzymes catalyzing the major steps in POMC processing are known, it is now possible to identify rate-limiting steps and control points governing this peptidergic system. The intermediate pituitary is innervated by dopaminergic neurons that project from the hypothalamus (Holzbauer and Racke, 1985; Davis, 1986). D_2-dopamine receptors, which play an important role in schizophrenia, Parkinson's disease, and tardive dyskinesia, are present at higher levels in the intermediate pituitary than in any other tissue (Bedard et al., 1986; Hoffman et al., 1989; Mengod et al., 1989; Mansour et al., 1990). To explore the mechanisms underlying dopaminergic regulation of POMC expression in the intermediate pituitary, rats have been treated chronically with either bromocriptine (a dopamine agonist) or haloperidol (a dopamine antagonist) (Hollt et al., 1982; Chen et al., 1983; Beaulieu et al., 1984; Kelsey et al., 1986; Bloomquist et al., 1991; Eipper et al., 1992). Melanoptrope biosynthesis of POMC and its product peptides was increased slightly after 21 days of treatment with haloperidol and decreased dramatically after chronic treatment with bromocriptine (Eipper et al., 1993). It is interesting that despite the >10-fold range in levels of POMC expression observed in bromocriptine- and haloperidol-treated animals, the same peptide products were produced in the same proportion. Recent data indicate that the expression of enzymes involved in POMC processing is regulated along with expression of POMC. Northern blot analysis of processing enzyme mRNAs after chronic drug treatment has shown that transcripts encoding PAM and CPH are up-regulated by haloperidol treatment and down-regulated by bromocriptine treatment (Birch et al., 1991; Bloomquist et al., 1991). It is interesting that PC1 and PC2 mRNA levels responded in the same manner as PAM, CPH, and POMC, indicating a coordinate regulation of peptide prohormone and processing enzyme mRNAs.

Based on studies involving a variety of stimuli, gene expression in peptidergic systems is also regulated on a time scale of hours (Sherman et al., 1986; Bhat et al., 1993). The melanotropes of the rat intermediate pituitary provide a relatively homogeneous cell system with which to investigate the effects of acute stimulation or inhibition by dopaminergic agents. In this study, we have investigated dopaminergic control of prohor-

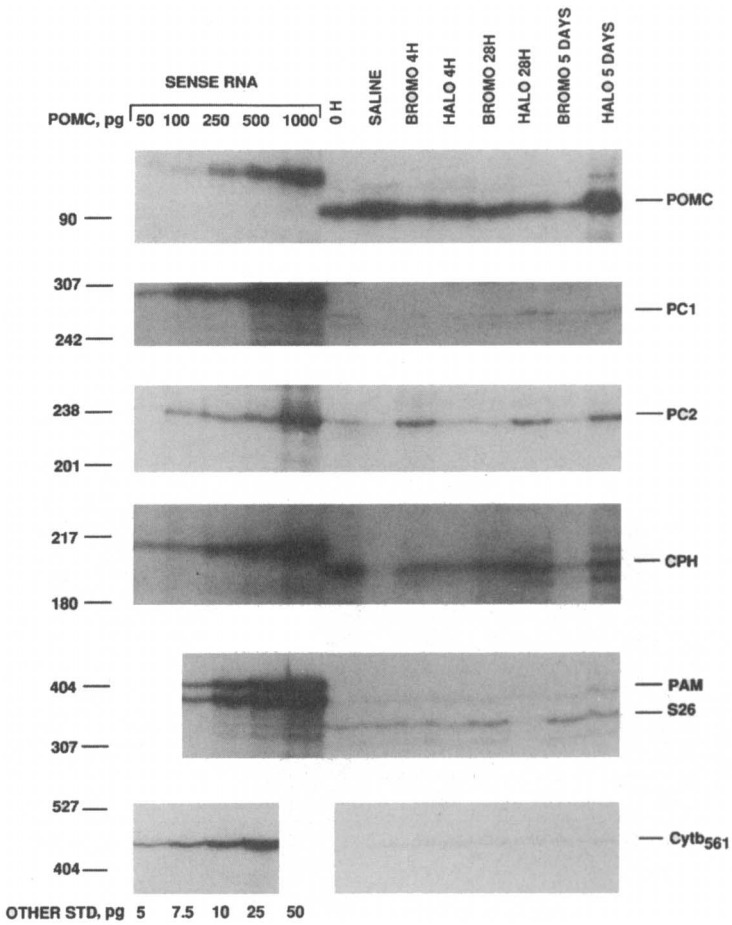


FIG. 2. RNase protection analysis of transcripts encoding granule-associated proteins. Total RNA isolated from neurointermediate pituitaries (n = 5 pituitaries/group) obtained from control rats (0 h), saline-treated rats, and rats treated once (4 h), twice (28 h), or five times with either bromocriptine (BROMO) or haloperidol (HALO) was assayed by RNase protection analysis for the presence of mRNA encoding POMC, PC1, PC2, CPH, PAM, and $Cytb_{561}$. S26 ribosomal mRNA was used as an internal control. Antisense riboprobes (^{32}P-labeled) were hybridized to 2.5–10 μg of total RNA or to known amounts of sense RNA and then digested with RNase as described in Materials and Methods. The protected fragments were fractionated on a 5% polyacrylamide gel containing 8 *M* urea and the size of each fragment was determined in reference to DNA markers (shown on left). The difference in the size of the sense and tissue protected pieces reflects the additional nucleotides present in the DNA template used for preparation of the probe and sense RNA. OTHER STD indicates the amounts of sense RNA used to determine the levels of PC1, PC2, PAM, CPH, $Cytb_{561}$, and S26 mRNAs.

mone and processing enzyme mRNA levels and biosynthetic rates by using RNase protection assays and metabolic labeling of melanotropes followed by immunoprecipitation. For a subset of the processing enzymes, dopaminergic regulation affects translation as well as levels of individual mRNAs.

MATERIALS AND METHODS

Drug treatments and RNA isolation

Adult (175–250 g) male Sprague–Dawley rats (Charles River, Wilmington, MA, U.S.A.) received five daily subcutaneous injections of bromocriptine (1 mg/kg/day), haloperidol (2 mg/kg/day), or saline (control) (Bloomquist et al., 1991). All protocols were approved by the Animal Care and Use Committee. Rats were killed by decapitation 1 or 4 h after four saline injections and a single injection of drug (1- and 4-h treatments), 4 h after three daily injections of saline followed by two daily injections of drugs (28 h), or 4 h after five daily injections of drug (5 day), and neurointermediate lobes were separated from anterior lobes under a dissecting microscope. Total RNA from neurointermediate lobes was isolated using the RNAgents Total RNA Isolation Kit (Promega, Madison, WI, U.S.A.). In brief, neurointermediate pituitary tissue was homogenized in guanidine thiocyanate and then subjected to phenol/chloroform/isoamyl alcohol extraction and isopropanol precipitation. RNA was quantitated by absorbance at 260 nm and analyzed by formaldehyde–agarose gel electrophoresis. An average of 5 μg of total RNA per neurointermediate lobe was obtained.

In vitro transcription of riboprobes

A set of seven cDNA fragments were subcloned into the pBluescript II SK− vector (Stratagene) by using either PCR or restriction endonucleases to remove the corresponding fragment from the original plasmid. The sizes of the cDNA inserts were selected to facilitate separation of the final protected RNA fragments by polyacrylamide gel electrophoresis (PAGE). The following cDNA fragments were subcloned by using restriction endonucleases and used as templates for preparation of probe RNAs: PAM–solution hybridization (PAM-SH) [385 nucleotides (nt), rPAM1(438–822 nt); insertion sites 5′-*Eco*RI and 3′-*Eco*RV] (Stoffers et al., 1989); POMC-SH [119 nt, rPOMC(149–267 nt); insertion sites 5′-*Apa*I and 3′-*Pst*I]; PC2-SH [202 nt, rPC2(1,388–1,589 nt); insertion sites 5′-*Eco*RI and 3′-*Xho*I] (Bloomquist et al., 1991); PC1-SH [277 nt, rPC1(2,140–2,416 nt); insertion sites 5′-*Apa*I and 3′-*Spe*I] (Bloomquist et al., 1991); and pBS.S26 [353 nt, S26(37–390 nt); insertion sites 5′-*Hin*dIII and 3′-*Bam*HI] (Kuwano et al., 1985). CPH [179 nt, rCPH(853–1,031 nt)] was amplified by PCR

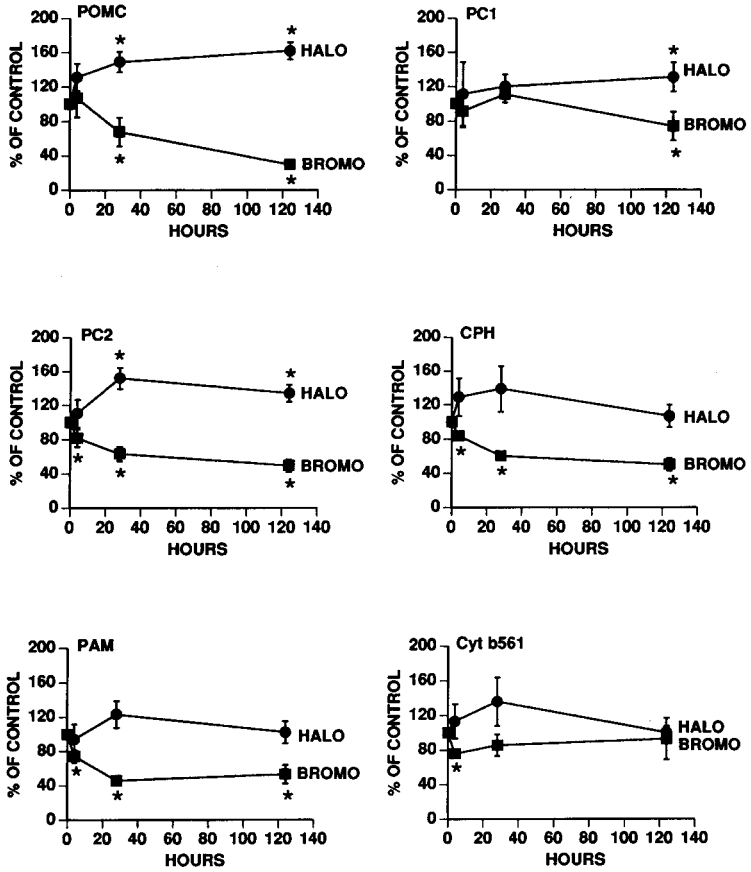


FIG. 3. Effect of dopaminergic agents on levels of POMC and processing enzyme mRNAs. The data obtained after RNase protection analyses like those shown in Fig. 2 were quantified using a PhosphorImager. The amount of each mRNA was normalized to the level of the internal control, S26 mRNA. The error bars represent mean ± SEM values of three independent experiments. Data points that differ significantly from control ($p < 0.05$) are indicated by asterisks. HALO, haloperidol; BROMO, bromocriptine.

from a 1.4-kb rat CPH probe in pSP65 (Fricker et al., 1989) using two oligonucleotides, a 27-mer **TCTAGA**CTGCTCACGAATACAGTTCCT that had an *Xba*I site (boldface) at the 5′ end and an antisense 27-mer **GGATCC**ACCGGGGACGCTGTACCATG containing a *Bam*HI site (boldface) at its 5′ end. In addition, $Cytb_{561}$-SH [442 nt, rat equivalent to $mCytb_{561}$(337–778 nt)] was amplified by reverse transcription–PCR of rat adrenal gland RNA using two oligonucleotides complementary to the mouse sequence (Srivastava et al., 1994), a 28-mer **GGATCC**GCACGTCTTCGCCTTCATCATC containing a *Bam*HI site (boldface) at the 5′ end and an antisense 26-mer **GAATTC**CATGGAGAGGGCTTGCTCTT with an *Eco*RI site (boldface) at its 5′ end. Each construct was verified by sequence analysis.

In preparation for the synthesis of sense RNA standards and antisense riboprobes, the plasmids were linearized either 5′ or 3′ to the cDNA insert, digested with 50 μg/ml proteinase K (Boehringer Mannheim) at 37°C for 30 min, and purified by phenol/chloroform extraction and ethanol precipitation. Linearized DNA templates (1 μg) were transcribed with 10 units of either T3 or T7 RNA polymerase, using an RNA transcription kit from Stratagene (La Jolla, CA, U.S.A.). For synthesis of high specific activity probes (1 to 8×10^8 cpm/μg), the transcription reaction was set up in a final volume of 25 μl containing 1× transcription buffer (200 m*M* Tris-HCl, pH 8.0, 40 m*M* $MgCl_2$, 10 m*M* spermidine, 250 m*M* NaCl), 0.4 m*M* each of rATP, rGTP, and rCTP, 0.004 m*M* UTP, 0.03 m*M* dithiothreitol, 40 units RNase inhibitor (Promega, Madison, WI, U.S.A.), and 50 μCi [α^{32}P]UTP (400–800 Ci/mmol, NEN, Boston, MA, U.S.A.). After incubation for 30 min at 37°C, 2 units RNase-free DNase I was added and the reaction mixture was incubated for another 15 min to remove plasmid DNA template. The reaction volume was increased to 70 μl with STE (20 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, 10 m*M* EDTA) and the radiolabeled transcript purified over a NucTrap column (Stratagene). POMC riboprobe was synthesized at a lower specific activity by using 0.04 m*M* rUTP in the reaction mixture. Probe size and integrity were checked on a 5% polyacrylamide gel containing 8 *M* urea.

Sense riboprobe standards for determining the levels of mRNA were synthesized as described above, but using 0.4 m*M* UTP; the amount of riboprobe was quantified by measuring absorbance at 260 nm after phenol/chloroform extraction and ethanol precipitation.

RNase protection assay

RNase protection assays were performed using the RPA II kit from Ambion (Austin, TX, U.S.A.). Total RNA (5–10 μg) and sense RNA (1–50 pg) were coprecipitated with $0.5–1 \times 10^6$ cpm of each ^{32}P-labeled riboprobe in the presence of 10 μg of yeast RNA and air dried. Pellets were resuspended in 20 μl of hybridization buffer (80% deionized formamide, 100 m*M* sodium citrate, pH 6.4, 300 m*M* sodium acetate, pH 6.4, 1 m*M* EDTA), heat denatured for 4 min at 90°C, and hybridized to probe (1×10^6 cpm) at 44°C over-

night. RNA digestion was performed at 37°C for 30 min after adding 200 μl of a mixture of 5 U/ml RNase A and 200 U/ml RNase T1 to the hybridization mix. Protected fragments were precipitated with 300 μl Ambion RNase inactivation/precipitation buffer using 10 μg of yeast tRNA and 60 μg of glycogen as carriers. RNA was resuspended in 8 μl of gel loading buffer, heat denatured at 90°C for 3 min, and fractionated on a 5% polyacrylamide gel containing 8 *M* urea. Gels were dried at 80°C under vacuum and exposed to film for 4–12 h. To quantify the levels of mRNA, the gels were digitized using a PhosphorImager (Molecular Dynamics). The size of each probe and protected fragment was determined by comparison with samples of pBR322 digested with *Msp*I (New England BioLabs) end labeled with [^{32}P]-dCTP using the Klenow enzyme (Boehringer Mannheim, Germany) for 30 min at 37°C, and purified over a NucTrap column.

Biosynthetic labeling of dispersed intermediate pituitary cells

After dissection, the neurointermediate lobes were transferred to Eppendorf tubes and dispersed in Dulbecco's modified Eagle medium (DMEM)-air containing 5 mg/ml trypsin (ICN Biochemicals, Cleveland, OH, U.S.A.) for 15 min at 37°C. After this incubation, trypsin was diluted with DMEM-air and the cells were collected by centrifugation at 100 *g* for 10 min. The dispersed cells were resuspended in methionine-free DMEM containing 1 mg/ml type II-L lima bean trypsin inhibitor and 2 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.), transferred to a new tube (leaving behind the neural lobes), and collected by centrifugation.

For biosynthetic labeling, dispersed intermediate pituitary cells were incubated in methionine-free complete serum-free medium (CSFM) containing 500 μCi [^{35}S]methionine/cysteine labeling mix (10 μ*M* methionine, 1,000 Ci/mmol; Amersham Corp., Arlington Heights, IL, U.S.A.) for 20 min in a 5% CO_2 atmosphere. CSFM contains 0.3 m*M* cysteine, meaning that essentially all of the label incorporated into proteins is [^{35}S]methionine; even in methionine/cysteine-free CSFM, incorporation of labeled [^{35}S]cysteine is negligible (Zhou and Mains, 1994). At the end of the pulse incubation with [^{35}S]methionine/cysteine, the cells were pelleted and extracted immediately in boiling sodium dodecyl sulfate–PAGE (SDS-PAGE) buffer (50 m*M* sodium phosphate, pH 6.8, containing 1% SDS, 50 m*M* β-mercaptoethanol, and 2 m*M* EDTA). Samples were heated to 95°C for 5 min and stored at −80°C. In pilot experiments, we labeled intact neurointermediate lobes from control or drug-treated animals; incorporation of [^{35}S]methionine into trichloroacetic acid precipitable material and into POMC was highly variable; acutely dispersed cells gave much more reproducible results.

Immunoprecipitations

After the SDS/β-mercaptoethanol extraction, a sevenfold excess of Nonidet P-40 over SDS was added and the samples were diluted fivefold with 50 m*M* sodium phosphate, pH 7.4, containing 1% Triton X-100 (Super E) plus phenylmethylsulfonyl fluoride (30 μg/ml). All samples were centrifuged at 14,000 *g* for 5 min to pellet-insoluble material and the supernatants were analyzed by immunoprecipitation.

The radiolabeled proteins were immunoprecipitated using rabbit polyclonal antisera against the following: peptidyl-α-hydroxyglycine α-amidating lyase (PAL), Ab877 [rPAM-1(498–604)]; amino-terminal region of ACTH (AbJH93); PC1, AbJH888[rPC1(359–373)]; and PC2, Ab1159[rPC2(626–638)] (Milgram et al., 1993; Zhou and Mains, 1994). The amount of antiserum necessary for quantitative immunoprecipitation of the proteins was determined by immunotitration of POMC, the most prevalent protein in melanotropes. After immunoprecipitation of labeled POMC with a fixed amount of antiserum, the supernatant was incubated with an additional amount of antiserum to immunoprecipitate any remaining POMC. No detectable POMC was left after the first immunoprecipitation, when 5 μl of antiserum was used with 1% of a neurointermediate lobe. An excess of antiserum (10 μl) was used to consistently immunoprecipitate each protein. Immune complexes were collected after incubation with 20 μl protein A–Sepharose beads (Sigma) in Super E. POMC was immunoprecipitated from 1% of the cell extract. The remainder of the sample was used for sequential immunoprecipitation of PC1, PC2, and PAM. The proteins were released from the beads by boiling into SDS sample buffer, and analyzed on 10% polyacrylamide, 0.25% *N,N'*-methylene-bisacrylamide–SDS gels (Laemmli, 1970). Gels were fixed in 30% isopropanol, 10% acetic acid, and incubated with Amplify (Amersham) for fluorography. Quantitative analysis of the samples was performed using a PhosphorImager (Molecular Dynamics).

Statistical analysis

For statistical analysis of mRNA levels and protein biosynthetic rates, the *t* test (two sample, one tail) was calculated using Excel 5.0.

RESULTS

The relatively homogeneous population of melanotropes in the neurointermediate lobe of the pituitary provides a model system for studying the coordinate regulation of enzymes involved in the processing of neuropeptides. Most of the processing enzymes are known and the chronic effects of dopaminergic treatment on POMC and several processing enzymes have been described (Birch et al., 1991; Bloomquist et al., 1991). We set out to evaluate the short-term effects of dopaminergic treatment on these peptidergic cells. The effects of bromocriptine and haloperidol treatments were evaluated by measuring levels of mRNA encoding POMC and peptidergic processing enzymes using RNase protection assays. The involvement of translational effects was evaluated by measuring the biosynthetic rates of POMC, PC1, PC2, and PAM.

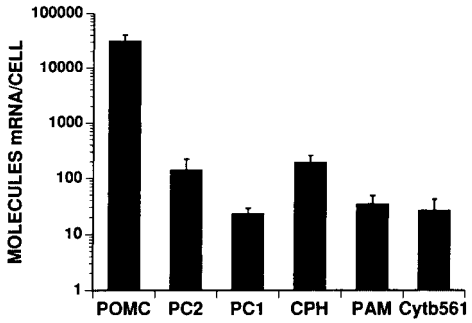
Transcript levels begin to change within a day of drug treatment

In previous studies, the levels of POMC mRNA approximately doubled after 21 days of haloperidol treatment (dopamine antagonist), whereas 21 days of treatment with a dopamine agonist caused a two- to fourfold diminution of POMC mRNA (Hollt et al., 1982; Chen et al., 1983; Kelsey et al., 1986; Bloomquist et al., 1991). Based on these findings, the time course of dopaminergic regulation of expression of a set of

mRNAs encoding POMC and peptidergic processing enzymes was studied in vivo. All animals received five daily injections, with drug treatments given five times (5 days), two times (28 h), once (4 or 1 h), or not at all (saline); control rats received no injections. Total RNA isolated from the neurointermediate lobes of these rats was subjected to multiplex RNase protection analysis by hybridizing one set of samples to POMC, PAM, and CPH riboprobes and another set of samples to PC1, PC2, and $Cytb_{561}$ riboprobes. The ribosomal protein S26 is expressed at comparable levels in many tissues (Vincent et al., 1993); analysis of S26 RNA in 5 μg RNA from control rats or rats treated with bromocriptine or haloperidol demonstrated no change in levels of S26 mRNA (data not shown). Thus, S26 riboprobe was also added to each sample as an internal control allowing us to measure simultaneously the levels of these transcripts and to correct for recovery. Representative autoradiographs demonstrating protected fragments of POMC and peptidergic processing enzymes are shown in Fig. 2. The sizes of each of the fragments obtained after RNase digestion were as expected. The assay was linear over the range of 50–1,000 pg of POMC RNA, 5–50 pg of the other RNAs, and was linear in the range of total RNA analyzed.

As shown in Fig. 3, significant changes in the levels of POMC mRNA were first observed after two injections (28 h sample) of bromocriptine with levels of POMC mRNA declining to about two-thirds of control values. After five daily injections of bromocriptine, levels of POMC transcript further decreased to about one-third of control. In contrast, haloperidol treatment caused a slight increase (maximum, 1.6-fold) in the levels of POMC mRNA, with little change between 28 h and 5 days. In previous studies (Hollt et al., 1982; Bloomquist et al., 1991), longer term (3 weeks) treatment of male rats with haloperidol caused at most, a twofold increase in POMC mRNA levels. Levels of POMC transcript were approximately fivefold higher in haloperidol versus bromocriptine-treated rats after 5 days.

Levels of peptidergic processing enzyme mRNA were determined after the same bromocriptine or haloperidol treatment paradigm. Significant decreases in levels of PC2, CPH, and PAM mRNA were detectable within 4 h of bromocriptine treatment. After 5 days of treatment with bromocriptine, levels of PC2, CPH, and PAM transcripts decreased to about one-half of control levels, whereas the levels of PC1 transcript were less affected and levels of $Cytb_{561}$ transcript were not significantly decreased. Haloperidol treatment caused a slight but significant increase in the levels of PC2 and PC1 mRNAs, whereas levels of PAM, CPH, and $Cytb_{561}$ mRNA were not significantly affected. Thus, treatment with bromocriptine brought about parallel decreases in the levels of POMC and PC2 mRNA, with PAM, CPH, and PC1 being less affected and $Cytb_{561}$ unaffected. Treatment with haloperidol had the opposite effect on levels of each of these transcripts. It is interesting that the data demonstrate coordinate regulation of peptide precursor and some, but not all, of the processing enzymes in the neurointermediate pituitary.



MOLECULES OF mRNA/CELL	31,000	140	23	191	34	26

FIG. 4. Level of POMC and processing enzyme mRNAs in control neurointermediate pituitaries. Total RNA was isolated from control neurointermediate lobes (n = 5) and subjected to multiplex RNase protection analysis and quantification with a PhosphorImager to determine the level of each transcript indicated. After quantification, the molecules of mRNA/cell were calculated using a yield of 5 μg RNA per neurointermediate lobe and published values of 3×10^5 melanotropes/adult male rat neurointermediate lobe (Mains et al., 1987; Bäck, 1989; Bäck and Soinila, 1994). Note logarithmic scale for the *y* axis. Each bar represents the mean ± SEM of five independent experiments.

Because RNase protection analysis provides an absolute measure of the amount of mRNA, we used the data from control animals to calculate the relative molar amounts of each transcript in the neurointermediate pituitary. As shown in Fig. 4, POMC transcripts were ~200-fold more prevalent than PC2 and CPH transcripts, and ~1,000-fold more prevalent than PAM, PC1, or $Cytb_{561}$ transcripts. Using our observed recovery of 5 μg total RNA per neurointermediate lobe and the literature values for the number of melanotropes per neurointermediate lobe (Mains et al., 1987; Bäck, 1989; Bäck and Soinila, 1994), we determined the number of molecules of mRNA/melanotrope. The number of POMC transcripts per cell is estimated to be 31,000, with less prevalent transcripts such as PC1 and PAM present in the range of 20–30 mRNA molecules per melanotrope. These results are consistent with the expression of both PC1 and PC2 in intermediate melanotropes, with PC2 being more abundant (Seidah et al., 1990; Bloomquist et al., 1991; Day et al., 1992).

Dopaminergic treatment has a translational effect on expression of POMC and processing enzymes

Although we observed that dopaminergic agents have an effect on mRNA levels, several observations indicated that dopaminergic agents might have an additional effect on the biosynthesis of POMC and its product peptides at the translational level as well (Beaulieu

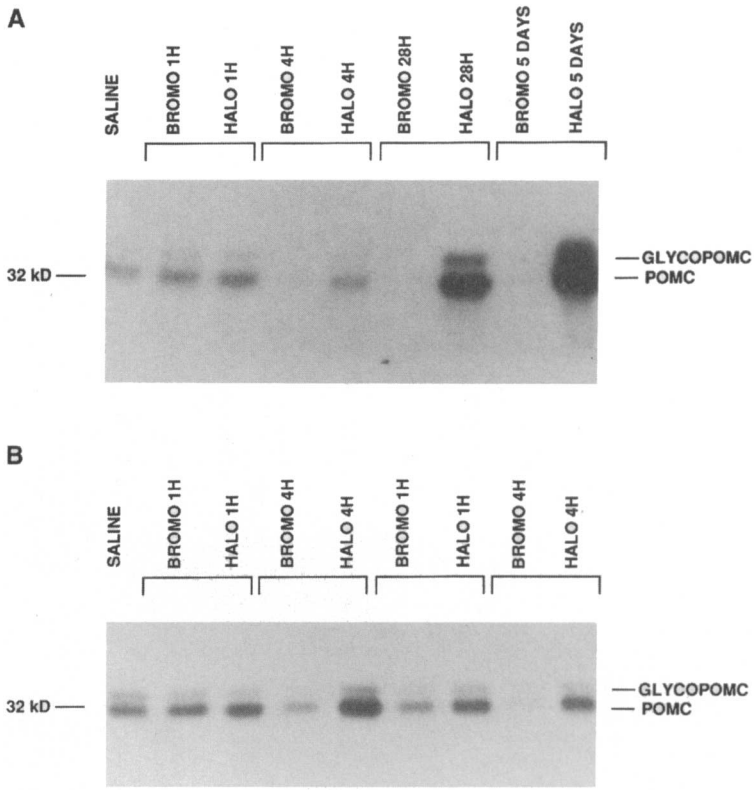


FIG. 5. Effect of bromocriptine (BROMO) or haloperidol (HALO) treatment on the biosynthesis of POMC. **A:** Melanotropes prepared from adult male rats treated with bromocriptine or haloperidol for the times indicated were labeled with [^{35}S]-methionine for 20 min and then extracted with SDS-PAGE buffer. Samples corresponding to 1% of the total cell extract were immunoprecipitated as described in Materials and Methods and fractionated on 10% SDS-polyacrylamide gels. Similar results were obtained in three independent experiments. **B:** Immunoprecipitation of POMC after single injections of drug following four injections of saline (1- and 4-h treatments), using nine sets of rats. Melanotropes were labeled and extracted as described above. Similar results were obtained in two independent experiments. The fluorographs were exposed to film for 3 weeks.

et al., 1984; Bloomquist et al., 1991; Levin and Roberts, 1991). To evaluate the effect of bromocriptine and haloperidol treatment on the rate at which POMC and processing enzymes are synthesized, dispersed melanotropes prepared from haloperidol- and bromocriptine-treated rats were incubated for 20 min in medium containing [^{35}S]methionine, extracted with boiling SDS-PAGE buffer, and immunoprecipitated with antisera to POMC, PC1, PC2, and PAM.

The two forms of POMC resolved by SDS-PAGE represent POMC molecules containing glycosylated and nonglycosylated ACTH. When the effect of treatment with bromocriptine was evaluated after 4 h, 28 h, or 5 days, a significant decrease in the biosynthetic rate of POMC was observed (Fig. 5A). To determine how rapidly the effect of bromocriptine could be observed, metabolic labeling studies were performed 1 and 4 h after a single injection of drug. Consistent decreases in the biosynthetic rate of POMC were observed as early as 4 h, but not 1 h, after a single injection of bromocriptine (Fig. 5B). It is interesting that the levels of POMC mRNA did not decrease significantly until the 28-h time point (Figs. 2 and 3), indicating that bromocriptine decreased the biosynthetic rate of POMC before changes in POMC mRNA were detectable. Haloperidol treatment caused a severalfold increase in the biosynthetic rate of POMC (Fig. 5A), an effect that was first observed at the 28-h time point and was far greater in magnitude than the effect of haloperidol on POMC mRNA levels (Fig. 3). A single injection of haloperidol did not produce consistent changes in the biosynthetic rate of POMC at 1 or 4 h after treatment. The translational effect of haloperidol treatment appeared at a later time than the translational effect of bromocriptine (4 h).

Similar effects on the biosynthetic rate of peptidergic processing enzymes were observed after bromocriptine and haloperidol treatment. The biosynthesis of endogenous or transfected PC1, PC2, and PAM in AtT-20 cells has been characterized previously (Eipper et al., 1993; Zhou et al., 1993; Zhou and Mains, 1994). The antisera used in these studies were used to immunoprecipitate PC2, PC1, and PAM from lysates of melanotropes. The antiserum to PC2 yielded a single band of 76-kDa proPC2, whereas antiserum to PC1 yielded proPC1 and PC1 at 87 and 81 kDa, respectively (Fig. 6). Three major forms of PAM mRNA occur in melanotropes, and PAM-1 (120 kDa), PAM-2 (105 kDa), and small amounts of newly synthesized PAM-3 were detected (Fig. 6A, B). Bromocriptine treatment consistently decreased the biosynthetic rate of PC2 at the 4-h time point but not at the 1-h time point (Fig. 6A). After a time course similar to the one observed for POMC biosynthesis, a significant effect of haloperidol treatment of the biosynthetic rate of PC2 was first observed consistently at the 28-h time point (Fig. 6B). Similar changes were observed in the biosynthesis of PC1 and PAM (Fig. 6B). The longer exposure times

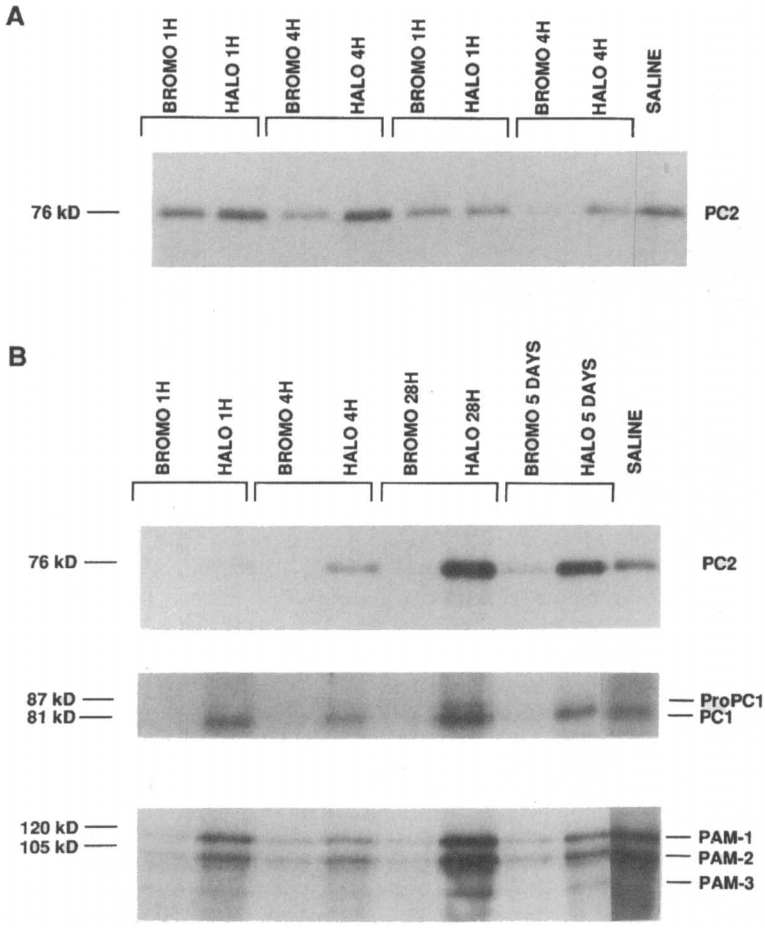


FIG. 6. Effect of bromocriptine (BROMO) or haloperidol (HALO) treatment on the biosynthesis of PC1, PC2, and PAM. Dispersed neurointermediate lobe melanotropes obtained from saline-treated rats and rats treated with bromocriptine or haloperidol were incubated with [^{35}S]methionine for 20 min and extracted with SDS-PAGE buffer. Samples were subjected to sequential immunoprecipitation with antisera against PC1, PC2, and PAM and analyzed by SDS-PAGE. **A:** Immunoprecipitation of PC2 after a single injection of drug (1 and 4 h). The fluorograph was exposed for 3 weeks. Similar results were obtained in three independent experiments. **B:** Immunoprecipitation of PC1, PC2, and PAM after bromocriptine and haloperidol treatment. The fluorographs were exposed for different periods of time: PC2, 3 weeks; PC1 and PAM, 5 weeks. Similar results were obtained in three independent experiments.

used for the fluorographs showing PC1 and PAM reflect the lower levels of these proteins in neurointermediate pituitary when compared with PC2 and POMC.

The biosynthetic rate of each immunoprecipitable protein after the drug treatments was quantified using a PhosphorImager. The biosynthetic rate of POMC was decreased to one-half of control levels 4 h after a single bromocriptine treatment (Fig. 7). After 28 h of bromocriptine treatment, the biosynthetic rate of POMC was further decreased to about one-fifth of control. The biosynthetic rate of PC2 was also decreased approximately twofold 4 h after a single injection of bromocriptine; however, no significant changes were observed at the 28-h time point after two drug treatments. Changes in PC1 biosynthesis were similar to the changes observed for PC2 (data not shown). The biosynthetic rate of PAM was decreased to two-thirds of control 4 h after a single injection of bromocriptine and was not further diminished after longer drug treatment (data not shown).

In rats treated with haloperidol for 28 h, the biosynthesis of POMC increased four- to sixfold despite only a 1.6-fold increase in POMC mRNA levels. Similar changes were observed for the biosynthetic rates of PC2 (Fig. 7), PC1, and PAM (data not shown). Haloperidol treatment affects the biosynthetic rate of POMC, PC1, PC2, and PAM much more dramatically than the mRNA levels; dopaminergic regulation of melanotropes involves a substantial translational effect.

By contrast, overall protein synthesis was not significantly affected by manipulation of the dopaminergic system. To ascertain the effect of the 28-h bromocriptine or haloperidol treatment on overall protein synthesis in melanotropes, the total extract after a 20-min incubation with [^{35}S]methionine was analyzed by two-dimensional PAGE. When the newly synthesized proteins from control and drug-treated intermediate pituitary cells were compared, only a subset of the proteins showed an effect of drug treatment on their synthesis. We compared the intensities of >100 well-resolved prevalent proteins in extracts of melanotropes from rats treated with bromocriptine or haloperidol (data not shown); the intensities of <10% of these proteins were significantly altered after haloperidol or bromocriptine treatment. It is interesting that three proteins showed a significant increase in synthesis with bromocriptine treatment and were not detectable in melanotropes from control animals or after treatment with haloperidol.

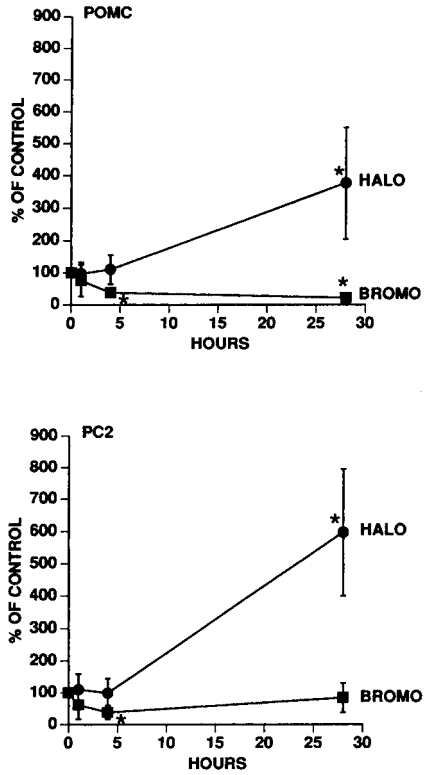
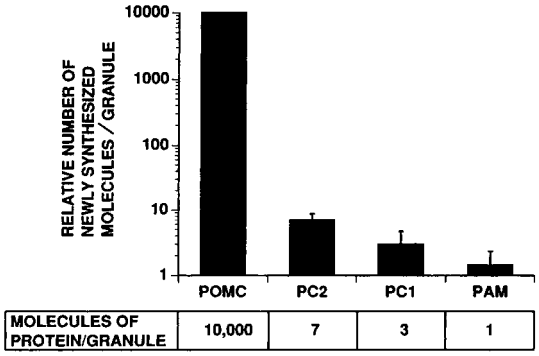


FIG. 7. Quantification of the biosynthetic rate of POMC and processing enzymes after drug treatment. Radiolabeled proteins immunoprecipitated from control and drug-treated melanotropes were fractionated on 10% SDS-polyacrylamide gels. Quantitative analysis of samples such as those shown in Fig. 5 was performed using a PhosphorImager. Relative biosynthetic rates were calculated. Similar results were obtained in three independent experiments. Data points that differ significantly from control ($p < 0.05$) are indicated by asterisks (*). HALO, haloperidol; BROMO, bromocriptine.

Because the methionine content of POMC, PC1, PC2, and PAM is known (Fig. 1), the molar ratio of these newly synthesized proteins was determined. In control neurointermediate pituitaries (Fig. 8), POMC molecules were synthesized ~1,500-fold more often than molecules of PC2, 3,000-fold more often than PC1 molecules, and 10,000-fold more often than PAM molecules. If one assumes that biosynthetic rates reflect steady-state levels of each protein, one can use literature values for secretory granule content of POMC products (10,000 molecules/granule) (Bäck, 1989) to calculate the relative amount of the other proteins per secretory granule. It is striking that none of the processing enzymes would occur at a level higher than 10 molecules per granule, with only one molecule of PAM per granule.

DISCUSSION

One key question addressed in this study about dopaminergic regulation in the intermediate pituitary is whether there is coordinate regulation of the expression of peptidergic processing enzymes. We first evaluated the time course with which treatment with bromocriptine and haloperidol affects the level of mRNA encoding several granule-associated proteins. The steady-state level of each mRNA reflects the rate of transcription and the rate of degradation. The response patterns fell into the following three categories: changes in levels of POMC and PC2 mRNA were the largest and were similar in magnitude; smaller changes were observed in levels of mRNA for PC1, CPH, and PAM mRNA; and $Cytb_{561}$ mRNA levels were unchanged. After treatment with bromocriptine or haloperidol for 3 weeks (Hollt et al., 1982; Bloomquist et al., 1991), POMC, PC2, and PAM mRNA levels showed changes similar in magnitude to the ones observed after 5 days of treatment; levels of PC1 mRNA changed twofold after the chronic treatment with haloperidol. It is interesting that levels of POMC, PC1, and PAM mRNA were also regulated in parallel in AtT-20 corticotrope tumor cells, with a decrease in all three transcripts after glucocorticoid treatment and an increase in all three after corticotropin-releasing factor treatment (Mains and Eipper, 1984; Thiele and Eipper, 1990; Bloomquist et al., 1991). Although levels of CPH mRNA were not affected by treatment with these secretagogues, CPH expression was subject to regulation by nicotine in chromaffin cells (Hook and Eiden, 1985), depolarization in PC12 cells (Das et al., 1992), and osmotic stimulation in oxytocin- and vasopressin-producing



MOLECULES OF PROTEIN/GRANULE	10,000	7	3	1

FIG. 8. Relative rates of synthesis of POMC and processing enzymes in control neurointermediate pituitary melanotropes. Neurointermediate pituitaries from four control adult male rats were dissociated and melanotropes were incubated with [^{35}S]-methionine for 20 min before extraction with SDS sample buffer. POMC immunoprecipitations were performed on 1% of the cell extract. The remainder of the sample was used for sequential immunoprecipitation of PC1, PC2, and PAM. After immunoprecipitation, the samples were fractionated by SDS-PAGE on a 10% gel and quantified using a PhosphorImager. Relative metabolic rates were calculated considering the number of methionines per protein (Fig. 1) and the amount of sample analyzed. As there are 3,000–10,000 molecules of POMC per granule (Bäck, 1989), the values were scaled to 10,000 molecules of POMC. Note the logarithmic scale for the *y* axis. The data shown represent mean ± SEM values of three independent experiments.

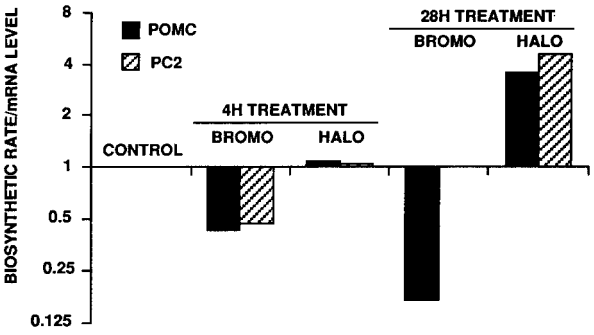


FIG. 9. Effect of drug treatment on ratio of protein synthesis to mRNA level for POMC and PC2. For better visualization of the early translational effects observed after treatment with bromocriptine (BROMO) or haloperidol (HALO), the relative ratios of protein synthesis to mRNA levels from three sets of experiments were determined using the data obtained after RNase protection analyses (Fig. 3) and metabolic labeling analyses (Fig. 7).

magnocellular neurons (Bondy et al., 1989). In general, changes in prohormone mRNA are greater in magnitude than changes in processing enzyme mRNAs.

Significant increases in the levels of POMC and processing enzyme mRNA were first observed 4 h after the second injection of haloperidol (28 h). No change in mRNA levels was observed 4 h after haloperidol treatment. Using dot blot hybridization, Chen et al. (1983) first detected an increase in POMC mRNA levels 6 h after haloperidol treatment. We observed that bromocriptine treatment brought about a significant decrease in levels of POMC and processing enzyme mRNA within 4 h. The half-life of POMC transcripts is 16–24 h (Roberts et al., 1979; Birnberg et al., 1983) and the half-life of PAM transcripts is ~20 h (Maltese et al., 1996). If transcription were halted immediately, levels of an mRNA whose half-life is 20 h would only decrease by 10% after 4 h. If bromocriptine affects mRNA levels by decreasing transcription without increasing turnover, the effects must be rapid.

Haloperidol treatment brought about a much bigger increase in the biosynthetic rate of POMC, PC1, PC2, and PAM proteins than predicted based on the mRNA levels. The increase in biosynthetic rate versus mRNA level was not apparent at 4 h but was clearly evident at 28 h (Fig. 9). For POMC and PC2, biosynthetic rates were increased approximately four times over that predicted by mRNA level after 28 h of haloperidol treatment. Thus, regulation by haloperidol occurs at the translational level as well as the transcriptional level. In addition, bromocriptine treatment produced a rapid decrease in the biosynthetic rates of the granule-associated proteins at 4 h after treatment (Fig. 7), and that decline in biosynthetic rate was not entirely explained by a decrease in mRNA levels (Fig. 9). After 28 h of bromocriptine treatment, biosynthetic rates more closely reflected mRNA levels. These results indicate that the response of melanotropes to bromocriptine and haloperidol involves early translational effects, in addition to longer term effects on mRNA levels.

We previously used the fact that long-term regulation by bromocriptine or haloperidol is specific to a subset of the proteins expressed in melanotropes to clone novel neuroendocrine proteins (Bloomquist et al., 1994). Our current metabolic labeling studies indicate that the short-term translational response of melanotropes to dopaminergic agents is also limited to a subset of the proteins expressed. Initiation of protein synthesis, the rate-limiting step in translation, is extensively regulated (Hershey, 1991; Kozak, 1991; Proud, 1994; Hentze, 1995). This process can involve the recognition of sequences and structural elements in specific mRNA molecules (McCarthy and Kollmus, 1995). A unique eight-nucleotide sequence present three times in the 5′-noncoding region of the preprosomastostatin II mRNA has been implicated as a regulatory site for interaction with putative *trans*-acting factors (Danoff and Shields, 1988). On the other hand, secondary structures in the 5′-untranslated region of mRNAs can control translation by modulating ribosome/mRNA interactions (Kozak, 1991). Structural mapping of the porcine POMC mRNA indicates that nucleotide sequences in the 5′-untranslated region can form a stable hairpin structure (Chevrier et al., 1988). Deletions of nucleotide sequences in the 5′-untranslated regions of the POMC and preprosomatostatin II mRNAs greatly increase the translational efficiency of these mRNAs. Insulin, which can stimulate overall translation rates as well as translation of specific mRNAs, stimulates phosphorylation of a protein that interacts with the translation initiation factor eIF-4E, inducing its dissociation and enhancing translation (Pause et al., 1994). Translation of mRNAs with a highly structured 5′-UTR can thus be selectively stimulated by insulin (Shantz and Pegg, 1994).

The coordinate regulation of a prohormone and its processing enzymes has also been observed in pancreatic β cells (Martin et al., 1994; Alarcon et al., 1993) and adrenal chromaffin cells (Laslop et al., 1994). Like POMC, insulin is synthesized as a prohormone and then processed to its active form by the action of PC1 and PC2 (Bailyes et al., 1991; Steiner et al., 1993*b*). It is interesting that the biosynthesis of both PC1 and PC2 is stimulated in parallel to the biosynthesis of proinsulin by elevated glucose (Alarcon et al., 1993; Martin et al., 1994). The stimulation of PC1 and proinsulin biosynthesis is not affected by the presence of actinomycin D, indicating that regulation occurs at a translational level (Alarcon et al., 1993). Although the translational effect of glucose on PC1 and proinsulin has been well documented, the mechanism underlying this regulation is still under investigation (Alarcon et al., 1993). In response to elevated glucose, β cells exhibit a rapid, coordinate increase in the bio-

synthesis of 25 of the 32 major granule proteins resolved on two-dimensional gels (Guest et al., 1991), indicating the specificity of glucose regulation. In rat adrenal chromaffin cells, reserpine increased the levels of mRNAs encoding proneuropeptide Y, secretogranin II, PC2, CPH, PAM, and chromogranin A and B; expression of $Cytb_{561}$ was not affected by reserpine (Laslop et al., 1994).

Studies examining the effect of exogenous processing enzyme expression clearly indicate that the level of expression is an important determinant of the cleavages observed (Bloomquist et al., 1991; Thomas et al., 1991; Seidah et al., 1993; Zhou et al., 1993). The intermediate pituitary is one of the few systems in which it is possible to obtain enzyme-to-substrate ratios. POMC mRNA is known to comprise ~10% of the mRNA in melanotropes (Bloomquist et al., 1991). Our data are consistent with this estimate. When the synthetic rate of POMC was compared with that of PC1, PC2, or PAM, we observed that POMC was synthesized 1,000-fold more often than PC2, 3,000-fold more often than PC1, and 10,000-fold more often than PAM. Given the prevalence of each of the mRNAs, the synthetic rates suggest that POMC (900 nt), PC2 (2,500 nt), PC1 (3,000 nt), and PAM (4,500 nt) mRNAs are translated with very similar efficiency. It is interesting that the ratio of POMC synthesis to synthesis of the most prevalent known endoprotease in melanotropes, PC2, is much higher than the ratio of POMC synthesis to PC1 synthesis in AtT-20 corticotrope tumor cells (five molecules of POMC for every one molecule of PC1 synthesized) (Zhou and Mains, 1994). The molar ratio of POMC to PAM molecules in AtT-20 cells was previously established to be ~3,000:1 (Mains and Eipper, 1990); this ratio is quite comparable with the ratio obtained in melanotropes. Because the amount of each processing enzyme is relatively low, treatments that selectively change enzyme level or enzyme activity may affect the level of active peptide produced (Bloomquist et al., 1991).

Our studies indicate that translational effects play a major role in the regulation of protein expression of processing enzymes in melanotropes in response to bromocriptine and haloperidol. The mechanisms by which these rapid translational effects are generated must be explored. Melanotropes in primary culture may be a useful tool in defining the mechanisms involved in the regulation of processing enzyme expression.

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