D-2 Dopamine Receptor-mediated Inhibition of Pro-opiomelanocortin Synthesis in Rat Intermediate Lobe

ABOLITION BY PERTUSSIS TOXIN OR ACTIVATORS OF ADENYlate CYCLASE*

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The melanotroph of the intermediate lobe of the rat pituitary gland contains mRNA directing the synthesis of pro-opiomelanocortin (POMC). POMC is processed into several smaller fragments including the melanotropic peptides (IR-aMSH) and /3-endorphin (1-5). A D-2 dopamine receptor occurs on the cells of the intermediate lobe (6-18). The D-2 dopamine receptor mediates inhibition of peptide hormone release from these cells. The D-2 dopamine receptor is believed to act through an inhibitory guanyl nucleotide-binding protein (termed N, (19, 20) or G, (21)) to decrease the release of peptide hormones. Pertussis toxin uncouples the D-2 dopamine receptor from N, and abolishes the ability of the receptor to mediate inhibition of adenylate cyclase activity or peptide release (22-35).

The present study was designed to investigate the mechanism by which the D-2 dopamine receptor inhibits POMC synthesis in rat intermediate lobe tissue. The involvement of the D-2 dopamine receptor in the synthesis and processing of POMC has been less well characterized than its involvement in the inhibition of hormone release. Dopaminergic drugs (when administered to rats for 24 h or longer) change the following parameters related to POMC synthesis by the intermediate lobe: the content of mRNA directing the synthesis of POMC; the capacity of the tissue to synthesize POMC; and the capacity of the tissue to synthesize IR-aMSH (36-38). Dopaminergic antagonists decrease these parameters while dopaminergic agonists increase these parameters. Furthermore, the intermediate lobes of stalk-sectioned animals have an enhanced capacity to synthesize POMC compared to the intermediate lobes from sham-operated animals (38). Taken together, these observations suggest that the dopaminergic neurons innervating the intermediate lobe exert a tonic inhibitory influence on the functioning of the melanotrophs. The biochemical mechanisms involved in the dopaminergic inhibition of POMC synthesis have not been investigated.

The present in vitro study was undertaken for the following reasons: 1) to determine whether dopaminergic drugs act...
directly on the intermediate lobe to diminish POMC synthesis, and 2) to determine whether Ni and adenylyl cyclase are involved in the dopaminergic inhibition of POMC synthesis. Isolated intermediate lobe tissue was incubated for up to 2 days under treatment protocols designed to elucidate the role of Ni and adenylyl cyclase in the dopaminergic inhibition of POMC synthesis. Following each treatment protocol, the tissue was tested for its capacity to incorporate [3H]tyrosine or [35S]methionine into POMC. Newly formed radiolabeled POMC was isolated by a newly developed procedure that employs disposable extraction columns.

EXPERIMENTAL PROCEDURES

Materials—Drugs and chemicals were obtained from the following sources: bromocriptine (CB154), Sandoz (East Hanover, NJ); 2-N-propyl-N-phenylethylamphetamine hydrobromide (N6893), gift from Alan S. Horn; trans-(-)-4-4a,4,5,6,7,8,9,9a-octahydro-5-propyl-1H pyrazolo[3,4-g]quinoline, monohydrochloride (LY171555), Eli Lilly & Co. (Indianapolis, IN); N-[2RS,3RS]-1-benzyl-3-pyriolidinyl-5-chloro-2-methoxy-4-methylpenitobenzamide (YM09151-2), Yamanauchi Pharmaceutical Co. (Tokyo, Japan); spiroperidol, Jaassen Pharmaceutica (New Brunswick, NJ); 7,8-dihydroxy-2,3,4,5-tetrahydro-1H-3-benzazepine, ethylamine, propyl-N-phenylethylamino)-5-hydroxy-tetralin (SKF38385), Smith Kline and French Laboratories (Philadelphia, PA); 3-hydroxytyramine (dopamine) and forskolin, Calbiochem-Behring. Pertussis toxin was prepared as described (39). 1-[3H]tyrosine (specific activity, 54 Ci/mmol) and 1-[35S]methionine (specific activity, 400 Ci/mmol), were from New England Nuclear. Selectamine, N+ and Ham's F10 medium from Grand Island Biological Co.; Eagle's minimum essential medium with Earle's salts, glutamine, horse serum, and fetal calf serum from Biofluids (Rockville, MD); bovine serum albumin (BSA, fraction V) Miles Laboratories, Inc.; and disposable extraction columns (CN, Catalog No. 7021-1) from J. T. Baker.

Preparation and Treatment of Tissue—After decapsulation of each rat (Sprague-Dawley, male, 300–350 g, Taconic Farms, Germantown, NY), intermediate lobe tissue was dissected from other pituitary tissue as previously described (12). Each intermediate lobe was collected in 1 ml of Eagle's Minimal Essential Medium fortified with 100,000 units/liter penicillin, 100 mg/liter streptomycin, HEPES (pH 7.4), and 0.25% bovine serum albumin (EMEM/BSA) equilibrated with a mixture of 95% air and 5% CO2. Each intermediate lobe was washed twice with 5 ml of EMEM/BSA and resuspended in 1 ml of Ham's F10 medium fortified with 2.5% fetal calf serum, 12.5% horse serum, 100,000 units/liter penicillin, and 100 mg/ml streptomycin equilibrated with a mixture of 95% air and 5% CO2. Each intermediate lobe was routinely incubated at 37°C in an atmosphere of 95% air and 5% CO2 in a multiwell plate in the absence or presence of the indicated drugs. When the effect of pertussis toxin was investigated (Fig. 3), intermediate lobe tissue was incubated in the absence and presence of the indicated concentrations of pertussis toxin for 18 h prior to the 24-h incubation in the presence of various other drugs (22, 39).

Assays—To determine the capacity of the intermediate lobe to synthesize POMC following various pretreatment protocols, intermediate lobe tissue was transferred to a Microfuge tube, centrifuged 5 s, and resuspended in 0.5 ml of EMEM/BSA without tyrosine (or without methionine, when appropriate) and incubated 30 min at 37°C (38). The tissue was then centrifuged, resuspended in 200 μl of EMEM/BSA containing 40 μg of [3H]tyrosine specific activity, 54 Ci/mmol; final concentration 3.7 μM) and incubated 30 min at 37°C. At the end of the incubation, each tube was centrifuged for 5 s; the resulting supernatant fluid was discarded, and the tissue was washed twice with 1-ml aliquots of complete EMEM/BSA. Intermediate lobe tissue was resuspended in 200 μl of N acetyl cysteine, containing 5 mg/ml bovine serum albumin and 0.17 mM phenylmethylsulfonyl fluoride and 0.17 mM iodoacetamide. This suspension was sonicated for 5 s and extracted overnight at 4°C. Occasionally, [35S]methionine (specific activity, 400 Ci/mmol; final concentration, 0.8 μCi) was substituted for the aid of a standard magnifying glass. Full size photographs are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-3882, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

Portions of this paper (including Table A and Figs. 1–111) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photographs are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-3882, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

RESULTS

D-2 dopaminergic agonists decreased the capacity of the intermediate lobe to synthesize POMC. Treatment of intermediate lobe tissue for 24 h with CB154 caused a dose-dependent inhibition in the capacity of the tissue to synthesize POMC (Fig. 2, left). Significant inhibition of POMC synthesis was obtained with incubations of 16 h or longer. For the sake of consistency with an earlier study (38), 24-h incubations were routinely used. The inhibitory effect of CB154 was blocked in a dose-dependent manner by spiroperidol, a D-2 dopaminergic antagonist (Fig. 2, right). N-0434 and LY171555, two selective D-2 dopaminergic agonists (44, 46), also inhibited POMC synthesis in a dose-dependent manner; YM09151-2, a selective D-2 antagonist (47), blocked the inhibitory effect of either agonist (Fig. 2, left, inset). SKF38385A, a selective D-1 dopamine agonist (48, 49), had no effect on the capacity of the intermediate lobe to synthesize POMC (Fig. 2, left, inset).

Pertussis toxin abolished the ability of D-2 agonists to inhibit POMC synthesis. Intermediate lobe tissue was treated with a range of concentrations of pertussis toxin for 18 h; the tissue was then incubated for an additional 24 h in the absence or presence of 0.1 μM CB154; finally, the capacity of the tissue to synthesize POMC was determined (Fig. 3). Pertussis toxin caused a dose-dependent abolition of the dopaminergic inhibition of POMC synthesis. In a parallel experiment, pertussis toxin was also tested for its ability to abolish the dopaminergic inhibition of adenylate cyclase activity. Intermediate lobe tissue was treated with a range of concentrations of pertussis toxin for 18 h. Each treatment group was washed, homogenized, and tested in the adenylate cyclase assay in the absence or presence of dopamine. The potency of pertussis toxin in attenuating the dopaminergic inhibition of adenylate cyclase activity was similar to the potency of pertussis toxin in attenuating the dopaminergic inhibition of POMC synthesis (Fig. 3, left, inset).

Compounds that activate the cAMP stimulatory pathway enhanced the capacity of the intermediate lobe to synthesize POMC. Intermediate lobe tissue was treated for 24 h with the indicated concentrations of either cholera toxin, forskolin, or 8-bromo-cAMP (Fig. 4). Each of these compounds caused a
dose-dependent increase in the capacity of the tissue to synthesize POMC. In addition, forskolin was tested for its ability to counteract the dopaminergic inhibition of POMC synthesis. Intermediate lobe tissue was incubated for 24 h in the absence of drugs (CONT), or in the presence of 30 nM CB154, 10 μM forskolin (FOR), or a combination of CB154 and forskolin. Four intermediate lobes were incubated separately for each treatment group. The capacity of the tissue to synthesize POMC was determined as described under “Experimental Procedures.” An aliquot of the sample of each group contained radioactivity closest to the mean of that group was subjected to electrophoresis on a 12% SDS-polyacrylamide gel as described under “Experimental Procedures.” The newly synthesized protein from each sample was isolated from disposable extraction columns as described under “Experimental Procedures.” The newly synthesized POMC was isolated from disposable extraction columns as described under “Experimental Procedures” and quantified by scintillation spectroscopy. Four determinations were made at each point; each determination was from a separate intermediate lobe. Data are expressed as mean ± S.E.

In three experiments, intermediate lobe tissue was treated for 24 h in the absence of drugs or in the presence of CB154, forskolin, or a combination of CB154 and forskolin. Four determinations were made at each point; each determination was from a separate intermediate lobe. Data are expressed as mean ± S.E.

A higher concentration of forskolin caused a greater enhancement of adenylate cyclase activity but failed to cause a further enhancement of POMC synthesis. Cholera toxin and 8-bromo-cAMP also counteracted the dopaminergic inhibition of POMC synthesis (Table I).

The intracellular and extracellular levels of IR-αMSH from intermediate lobe tissue treated for 24 h in the absence of drugs or in the presence of CB154, forskolin, or a combination of CB154 and forskolin are shown in Table II. All drug-treated groups had elevated tissue levels of IR-αMSH compared to control levels. Treatment with CB154 alone decreased the level of extracellular IR-αMSH, whereas treatment with either forskolin alone or in combination with CB154 increased the level of extracellular IR-αMSH compared to the control level.

In three experiments, intermediate lobe tissue was treated for 24 h in the absence of drugs, or in the presence of CB154, forskolin, or a combination of CB154 and forskolin (Fig. 5). The level of POMC mRNA in each group was then quantified by hybridization with a radiolabeled probe derived from bovine tissue (see Miniprint Supplement). Tissue treated with CB154, forskolin, or a combination of CB154 and forskolin increases...
pertussis toxin. The tissue was then incubated in the absence of pertussis toxin. During the last 2 h of incubation, cholera toxin (30 nM) was added to all incubation samples described under "Experimental Procedures" either in the absence or presence (filled circles) of 0.1 μM bromocriptine. The capacity of the tissue to synthesize POMC was determined as described in the legend to Fig. 2. Four determinations were made at each point; each determination was made on a separate intermediate lobe. Data are expressed as mean ± S.E. Inset, intermediate lobe tissue was incubated for 18 h in the presence of the indicated concentrations of pertussis toxin (five lobes for each concentration of pertussis toxin). During the last 2 h of incubation, cholera toxin (30 nM) was added to all incubation samples (22). Adenylate cyclase activity (cyclase) was assayed in a homogenate of each treatment group as described under "Experimental Procedures" either in the absence (open circles) or presence (filled circles) of 100 μM dopamine. Adenylate cyclase activity is expressed as picomoles of cAMP/mg of protein/min. Data represent mean ± S.E. (n = 4) obtained in a single experiment. This experiment was performed two additional times with similar results.

had 54, 320, and 178% of the control level of POMC mRNA, respectively. The changes in amount of POMC mRNA were not the result of differences in the amount of tissue RNA, since equal amounts of total RNA were applied to each lane of the gel.

DISCUSSION

The present study presents evidence suggesting that the D-2 dopamine receptor in the intermediate lobe of the rat pituitary gland regulates the capacity of this tissue to synthesize POMC (44-49). The D-2 dopamine receptor in the intermediate lobe is associated with an inhibitory guanyl nucleotide-binding protein (termed N, (19)) (12, 13). N, acts as a transducer converting stimulation of the D-2 receptor into inhibition of adenylate cyclase activity (19). Pertussis toxin uncouples N, from the D-2 dopamine receptor and abolishes the receptor-mediated inhibition of adenylate cyclase activity (22). In the present study, pertussis toxin caused a dose-dependent abolition of the dopaminergic inhibition of POMC synthesis. This finding strongly suggests that N, and cAMP participate in the dopaminergic inhibition of POMC synthesis.

This study also presents evidence that N,, the stimulatory guanyl nucleotide binding protein (19), adenylate cyclase, and cAMP participate in the regulation of capacity of the inter-

FIG. 3. Pertussis toxin abolishes the D-2 dopaminergic inhibition of POMC synthesis. Intermediate lobe tissue was incubated for 18 h in the presence of the indicated concentrations of pertussis toxin. The tissue was then incubated in the absence (open circles) or the presence (filled circles) of 0.1 μM bromocriptine. The capacity of the tissue to synthesize POMC was determined as described in the legend to Fig. 2. Four determinations were made at each point; each determination was made on a separate intermediate lobe. Data are expressed as mean ± S.E. Inset, intermediate lobe tissue was incubated for 18 h in the presence of the indicated concentrations of pertussis toxin (five lobes for each concentration of pertussis toxin). During the last 2 h of incubation, cholera toxin (30 nM) was added to all incubation samples (22). Adenylate cyclase activity (cyclase) was assayed in a homogenate of each treatment group as described under "Experimental Procedures" either in the absence (open circles) or presence (filled circles) of 100 μM dopamine. Adenylate cyclase activity is expressed as picomoles of cAMP/mg of protein/min. Data represent mean ± S.E. (n = 4) obtained in a single experiment. This experiment was performed two additional times with similar results.

FIG. 4. Compounds that activate the cAMP pathway in the intermediate lobe enhance the capacity of the tissue to synthesize POMC. Intermediate lobe tissue was incubated for 24 h in the presence of the indicated concentrations of cholera toxin (open circles), forskolin (filled circles), or 8-bromo-cAMP (open diamonds). The capacity of the intermediate lobe tissue to synthesize POMC was determined as described in the legend to Fig. 2. Four determinations were made at each point; each determination was made on a separate intermediate lobe. Data were obtained in three separate experiments. POMC synthesis in the absence of drugs is expressed as 100%; POMC synthesized on the presence of drugs is expressed as a per cent of control values.

mediate lobe to synthesize POMC. The participation of N, in the regulation of POMC synthesis by the intermediate lobe is suggested by the ability of cholera toxin to increase POMC synthesis with approximately the same molar potency that it displays as a stimulant of adenylate cyclase (13, 49–56). The involvement of adenylate cyclase per se in the regulation of POMC synthesis is suggested by the ability of forskolin to increase POMC synthesis and to overcome the D-2 dopaminergic inhibition of POMC synthesis. The direct involvement of cAMP in the regulation of POMC synthesis is suggested by the ability of 8-bromo-cAMP to mimic the effects of cholera toxin (52). The involvement of adenylate cyclase in the dopaminergic inhibition of POMC synthesis is suggested by the ability of forskolin and cholera toxin to reverse the dopaminergic inhibition of POMC synthesis at concentrations that reverses the dopaminergic inhibition of adenylate cyclase activity.

The findings of the present study provide evidence for the following model of the dopaminergic regulation of POMC synthesis in the rat melanotroph: Stimulation of the D-2 receptor on the surface of the melanotroph alters the property of N, so that intracellular GTP can activate N, causing N, to inhibit adenylate cyclase activity and thereby lower the intracellular concentration of cAMP. The diminished level of cAMP results in a decrease in the capacity of the cell to synthesize POMC. Diminished gene transcription provides one potential mechanism for decreasing POMC synthesis as a consequence of stimulating the D-2 receptor: in vivo studies, CB154 caused parallel decreases in the content of POMC mRNA and in the capacity of the tissue to synthesize POMC (57, 38). In the current study (Fig. 6), treatment of intermediate lobe tissue with CB154 for 24 h resulted in a
Forskolin reverses the D-2 dopaminergic inhibition of POMC synthesis. Intermediate lobe tissue was incubated for 24 h in the absence of drugs, or in the presence of 30 nM CB154, 10 μM forskolin, or a combination of 30 nM CB154 and 10 μM forskolin as described under "Experimental Procedures." The amount of IR-aMSH in the tissue (intracellular) and in the media (extracellular) were determined by radioimmunoassay as described under "Experimental Procedures." Four intermediate lobes were incubated separately for each treatment group. Data are expressed as mean ± S.E.

Table I
Activators of the cAMP pathway counteract the D-2 dopaminergic inhibition of POMC synthesis.

| Drug            | POMC synthesis |
|-----------------|----------------|
| Drug alone      | Drug plus 0.1 μM N-0434 |
| None            | 105 ± 8 | 60 ± 1 |
| Forskolin (10 μM) | 163 ± 13 | 177 ± 11 |
| Cholera toxin (0.1 μM) | 148 ± 2 | 158 ± 13 |
| 8-bromo-cAMP (10 mM) | 151 ± 6 | 147 ± 8 |

Fig. 5. Forskolin reverses the D-2 dopaminergic inhibition of POMC synthesis. Intermediate lobe tissue was incubated for 24 h in the absence of drugs, or in the presence of 30 nM CB154, 10 μM forskolin, or a combination of 30 nM CB154 and 10 μM forskolin as described under "Experimental Procedures." The amount of IR-aMSH in the tissue (intracellular) and in the media (extracellular) were determined by radioimmunoassay as described under "Experimental Procedures." Four intermediate lobes were incubated separately for each treatment group. Data are expressed as mean ± S.E.

Table II
Effect of CB154, forskolin, or a combination of CB154 and forskolin on intermediate lobe IR-aMSH content and release

| Drug            | Intracellular | Extracellular |
|-----------------|---------------|---------------|
| Control         | 54 ± 4 | 24.0 ± 2.4 |
| CB154           | 104 ± 16.2 | 14.0 ± 2.0 |
| Forskolin       | 80 ± 12.2 | 49.0 ± 5.0 |
| CB154 and forskolin | 79 ± 7.1 | 44.4 ± 3.2 |

Fig. 6. Changes in tissue levels of POMC mRNA following treatment with CB154, forskolin, or a combination of CB154 and forskolin. Intermediate lobe tissue (10 lobes/group) were incubated for 24 h in the absence of drugs, or in the presence of either CB154 (30 nM), forskolin (10 μM), or a combination of CB154 (30 nM) and forskolin (10 μM) as described under "Experimental Procedures." Total RNA was extracted, and tissue levels of POMC mRNA were determined by Northern blot analysis as described in text. The amount of POMC mRNA hybridized was quantified by cutting out the sections of the gel corresponding to the location of the bands on the x-ray film and determining the radioactivity of each section by liquid scintillation spectroscopy. This experiment was repeated three times. The actual amounts of radioactivity ± S.E. in the three experiments were 740 ± 18, 396 ± 16, 2366 ± 79, and 1316 ± 37 for the control, CB154-treated, forskolin-treated, and CB154 plus forskolin-treated groups, respectively. Kb, kilobase.
and an atrophy of intermediate lobe tissue (38, 61). Taken together, these observations suggest that activation of the dopamine receptor does not merely diminish transcription of the POMC gene but rather causes a coordinated diminution of numerous processes involved in the synthesis of the intermediate lobe peptides.

While this paper was being reviewed for publication, a similar study showing that dopamine inhibited pro-opiomelanocortin synthesis in the amphibian pituitary intermediate lobe appeared in this journal (68).

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Analyzing the protein composition and the determination of the intermediate lobe tissue samples from POMC synthesis in Rat Intermediate Lobe.

An intermediate lobe tissue was isolated and homogenized in the presence of 0.1 M Tris- HCl, pH 7.4, and homogenates were applied to disposable extraction columns (Cn) as described in Methods. Rat intermediate lobe tissue was applied to each column at pH 7.4, and 5 mg of tissue was added to each column in 10 ml of phosphate buffer (pH 7.4). The columns were then washed with 5 ml of phosphate buffer (pH 7.4) and the wash fractions were collected together to give the sum of washes. The tissue was then treated with 5 ml of cold 0.05% Triton X-100 (5°C) for 30 min, and the cold wash fractions were collected together to give the sum of washes. The tissue was then treated with 5 ml of cold 0.05% Triton X-100 (5°C) for 30 min, and the cold wash fractions were collected together to give the sum of washes.

A similar aliquot of each of the wash fractions was subjected to electrophoresis. The recovery of radioactive material applied to the column was 87 ± 3% (mean ± SEM, n = 5).

Figure 11. Apparent purity of radioactive material from disposable extraction column.

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Table A. Characterization of radioactive material isolated by chromatographic techniques.

| Column Extract | Apparent Mobility | % of Control |
|----------------|------------------|-------------|
| (kDa)          | (kDa)            | (kDa)       |
| 70             | 70               | 70          |
| 35             | 35               | 35          |
| 18             | 18               | 18          |
| 11             | 11               | 11          |

Table B. Apparent purity of radioactive material from disposable extraction column.

| Column Extract | Apparent Mobility | % of Control |
|----------------|------------------|-------------|
| (kDa)          | (kDa)            | (kDa)       |
| 70             | 70               | 70          |
| 35             | 35               | 35          |
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| 35             | 35               | 35          |
| 18             | 18               | 18          |
| 11             | 11               | 11          |