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Hypoprolactinemia decreases tyrosine hydroxylase activity in the tuberoinfundibular dopaminergic neurons acutely by protein dephosphorylation and chronically by changes in gene expression

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This study evaluated the roles of protein dephosphorylation or suppressed gene expression in reducing tyrosine hydroxylase activity in tuberoinfundibular dopaminergic neurons after acute or chronic bromocriptine (BROMO) administration. Diestrous or ovariectomized rats were injected with BROMO (3 mg/kg, s.c.) at 1000 h and were sacrificed 4 h later or were injected with BROMO at 12 h intervals for 3 days. *In vitro* tyrosine hydroxylase activity was assessed by incubating hypothalamic explants with brocresine, an L-aromatic amino acid decarboxylase inhibitor, and measuring 3,4-dihydroxyphenylalanine (DOPA) accumulation in the stalk-median eminence (SME). The incubation medium also contained either 2 μM okadaic acid, a protein phosphatase 1 and 2A inhibitor, or its vehicle (0.25% dimethylsulfoxide). Acute (4 h) and chronic (3 days) BROMO treatment suppressed circulating PRL levels from 10–12 ng/ml to < 1 ng/ml and reduced tyrosine hydroxylase activity in the SME by 60% or 40% in diestrous or ovariectomized rats, respectively. Okadaic acid increased tyrosine hydroxylase activity in the SME 2-fold in control diestrous or ovariectomized rats. The reduced tyrosine hydroxylase activity in the SME after acute BROMO treatment was increased by okadaic acid 5- or 3-fold in diestrous or ovariectomized rats respectively, to a value similar to the controls. In sharp contrast, after chronic BROMO treatment, the response to okadaic acid was blunted. As assessed by *in situ* hybridization, tyrosine hydroxylase mRNA signal levels in the arcuate nucleus of diestrous rats were not altered after acute BROMO treatment, but were reduced by 70% after chronic BROMO treatment. The acute BROMO-induced decrease in tyrosine hydroxylase activity was reversed by co-administration of oPRL or rPRL, indicating that the action of BROMO is via a reduction in PRL. The data suggest that protein dephosphorylation may be a primary mechanism for the rapid BROMO-dependent suppression of tyrosine hydroxylase activity, whereas suppression of tyrosine hydroxylase gene expression may contribute to the lower tyrosine hydroxylase activity after chronic BROMO treatment.

Keywords: tyrosine hydroxylase; dopamine, prolactin; tuberoinfundibular; hypoprolactinemia; bromocriptine; phosphorylation

Introduction

The tuberoinfundibular dopaminergic neurons have cell bodies in the arcuate nucleus and nerve terminals in the median eminence (Björklund *et al.*, 1973). Dopamine released from these neurons acts as a major PRL inhibiting hormone (Ben-Jonathan, 1994). PRL, in turn, exerts a negative feedback on its release, in part, by altering the synthesis and secretion of dopamine, such that conditions of hyperprolactinemia or hypoprolactinemia increase or decrease neuronal activity, respectively (Moore, 1987). Indeed, the functional integrity and normal development of the tuberoinfundibular dopaminergic neurons is dependent on a finite amount of PRL (Demarest *et al.*, 1985; Morgan & King, 1986; Shyr *et al.*, 1986; Arbogast & Voogt, 1991; Romero & Phelps, 1993). The PRL feedback involves both a rapid 'tonic' component, which is unmasked when circulating levels of endogenous PRL are suppressed by bromocriptine (BROMO), a dopamine agonist, and a delayed 'induction' component which sustains the capacity of these neurons to respond to PRL (Demarest *et al.*, 1984). In contrast to the tuberoinfundibular dopaminergic neurons which respond to dopaminergic antagonists and agonists because of their ability to alter PRL release (Demarest & Moore, 1980; Gudelsky & Porter, 1980; Arita & Kimura, 1986; Arbogast & Voogt, 1991), the incerto-hypothalamic and nigrostriatal dopaminergic neurons appear to respond to these agents by an autoreceptor regulatory mechanism (Kehr *et al.*, 1972; Demarest & Moore, 1979, 1980; Lookingland & Moore, 1984; Galloway *et al.*, 1986). The incerto-hypothalamic dopaminergic neurons are in the medial zone incerta (Björklund *et al.*, 1975). The major ascending dopaminergic neuronal systems have their perikarya in the substantia nigra and ventral tegmentum (Dahlström & Fuxe, 1964).

Tyrosine hydroxylase is the rate limiting enzyme in the dopaminergic biosynthetic pathway (Levitt *et al.*, 1965). The activity of tyrosine hydroxylase can be regulated by activation (e.g. phosphorylation) of the existing enzyme or synthesis/degradation of enzyme molecules (Masserano & Weiner, 1983). The intracellular mechanism(s) which contributes to the PRL control of tyrosine hydroxylase activity in the tuberoinfundibular dopaminergic neurons is not completely understood. Although elevated PRL levels increase tyrosine hydroxylase activity in the median eminence, tyrosine hydroxylase mRNA levels in the arcuate nucleus and enzyme quantity in the median eminence are increased under some, but not all, conditions of hyperprolactinemia (Gonzalez & Porter, 1988; Arbogast & Voogt, 1991; Selmanoff *et al.*, 1991). Thus, it is unlikely that changes in the expression of the tyrosine hydroxylase gene is the sole mechanism for the hyperprolactinemia-induced increase in enzyme activity. However, a finite amount of PRL appears to be essential for normal function in dopamine neurons in the arcuate nucleus, in part by sustaining tyrosine hydroxylase mRNA levels (Arbogast & Voogt, 1991). We previously reported that 3 days of BROMO treatment reduced both tyrosine hydroxylase activity in the stalk-median eminence (SME) and mRNA levels in the arcuate nucleus, but not the substantia nigra and zona incerta, and that this effect was reversed by co-administration of PRL (Arbogast & Voogt, 1991). However, BROMO suppresses tyrosine hydroxylase activity within hours (Demarest *et al.*, 1984) and it is unclear if changes in gene expression or protein dephosphorylation contribute to the early events.

The objectives of this study were: (1) to examine the catalytic activity of tyrosine hydroxylase in the SME and striatum after acute (4 h) or chronic (3 days) BROMO treatment and to relate changes in tyrosine hydroxylase activity to circulating PRL levels; (2) to evaluate the role of protein dephosphorylation in the BROMO-induced reduction in

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tyrosine hydroxylase activity in the SME by studying the effect of okadaic acid, an inhibitor of phosphoprotein phosphatases 1 and 2A, on tyrosine hydroxylase activity after acute or chronic BROMO treatment; (3) to determine tyrosine hydroxylase mRNA signal levels in the arcuate nucleus and zona incerta after acute or chronic BROMO treatment, and (4) to investigate the PRL-dependence of the acute BROMO-induced reduction in tyrosine hydroxylase activity by co-administration of oPRL or rPRL.

Results

Effect of okadaic acid on tyrosine hydroxylase activity in the SME and striatum after bromocriptine treatment

The first experiment evaluated the ability of okadaic acid to increase tyrosine hydroxylase activity in the SME and striatum in diestrous and ovariectomized rats after acute (4 h) and chronic (3 days) BROMO treatment. In diestrous rats, tyrosine hydroxylase activity in the SME decreased ($P<0.05$) by 60% within 4 h after BROMO injection and was similarly low after 3 days of BROMO treatment (Figure 1; top panel). Inclusion of okadaic acid in the incubation medium ($P<0.05$) increased tyrosine hydroxylase activity in the SME approximately 100% in vehicle-treated control rats. Okadaic acid increased enzyme activity 500% after acute BROMO treatment to levels similar to those observed in the control rats. In contrast, okadaic acid increased ($P<0.05$) tyrosine hydroxylase activity in the SME by only 60% after chronic BROMO treatment and the magnitude of the increase was markedly reduced from that observed after acute BROMO treatment. Tyrosine hydroxylase activity in the striatum was similar at 4 h and 3 days after BROMO treatment and okadaic acid caused a similar 2-fold increase in all groups (Figure 1; bottom panel). Serum PRL concentrations (ng/ml) were suppressed from 9.8 ± 2.5 (Vehicle) or 11.8 ± 1.9 (okadaic acid) in control rats to 0.7 ± 0.06 (vehicle) or 0.7 ± 0.08 (okadaic acid) 4 h after BROMO treatment and to 0.7 ± 0.06 (vehicle) or 0.8 ± 0.09 (okadaic acid) after 3 days of BROMO treatment.

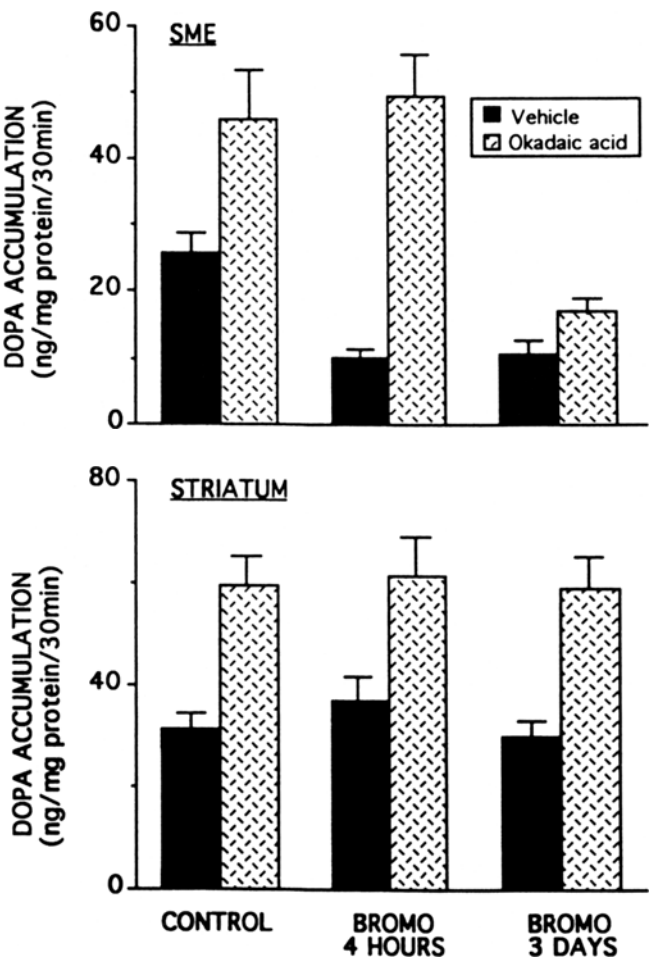

Figure 1 *In vitro* DOPA accumulation in the SME (top panel) and striatum (bottom panel) in diestrous rats after acute (4 h) or chronic (3 days) BROMO treatment. Okadaic acid (2 μM) or its vehicle (0.25% dimethylsulfoxide) were included in the medium as indicated. *In vitro* DOPA accumulation in the SME was reduced ($P<0.05$) after 4 h or 3 days of BROMO treatment. The magnitude of the okadaic acid-induced increase in tyrosine hydroxylase activity in the SME was greater ($P<0.05$) in control and 4 h BROMO-treated groups than in the 3 days BROMO-treated group. BROMO treatment did not alter basal DOPA accumulation in the striatum or the response to okadaic acid. Each value is a mean ± SE of determinations from 10–11 rats

In ovariectomized rats, the experiments with acute and chronic BROMO treatment were performed at different times. Acute and chronic BROMO treatment reduced ($P<0.05$) tyrosine hydroxylase activity in the SME by 35% and 30%, respectively (Table 1). Okadaic acid increased enzyme activity to similar levels in control and BROMO-treated after 4 h. After 3 days of BROMO treatment, okadaic acid increased tyrosine hydroxylase activity only 2-fold, as compared to a 4-fold increase in control rats. Serum PRL levels were markedly suppressed after both 4 h and 3 days of BROMO treatment (Table 1).

Effect of acute and chronic bromocriptine treatment on tyrosine hydroxylase mRNA

Tyrosine hydroxylase mRNA levels in the arcuate nucleus were not altered 4 h after BROMO injection, but were reduced ($P<0.05$) to 30% of control levels after 3 days of BROMO treatment (Figure 2; top panel and Figure 3; top panel). The number of detectable tyrosine hydroxylase mRNA-containing cells in the arcuate nucleus were 21.9 ± 0.9, 21.9 ± 0.7 and 23.4 ± 0.8 cells/section in control, BROMO-4 h and BROMO-3 days, respectively. The intensity of tyrosine hydroxylase immunostaining in the arcuate-median eminence region was similar to controls 4 h after BROMO treatment, but was reduced after 3 days of BROMO treatment (Figure 2; bottom panel). The cellular content of tyrosine hydroxylase mRNA in the zone incerta was similar in all groups (Figure 3; bottom panel).

Reversal of the acute bromocriptine effect by PRL replacement

This experiment examined the ability of PRL replacement to reverse the BROMO-induced decrease in tyrosine hydroxylase activity. In diestrous rats, tyrosine hydroxylase activity in the SME was reduced ($P<0.05$) by 40% 4 h after BROMO treatment (Figure 4; top panel). Replacement of PRL by co-administration of either oPRL or rPRL prevented the BROMO-induced decrease in enzyme activity, whereas

Table 1 Tyrosine hydroxylase activity in the SME and circulating PRL levels in ovariectomized rats after acute (4 h) and chronic (3 days) bromocriptine treatment

	DOPA accumulation (ng/mg protein/30 min		
	Vehicle	*Okadaic acid*	*Serum PRL (ng/ml)*
Control-4 h	13.6 ± 2.6	24.1 ± 4.8	4.5 ± 0.6
BROMO-4	8.8 ± 0.8*	25.5 ± 4.9	0.6 ± 0.1*
Control-3 days	14.4 ± 1.6	56.4 ± 6.4	10.2 ± 1.2
BROMO-3 days	8.8 ± 0.9*	19.4 ± 2.9*	1.0 ± 0.1*

Each value is a mean ± SE of determinations from 9–11 rats. *Significantly ($P<0.05$) different from respective control

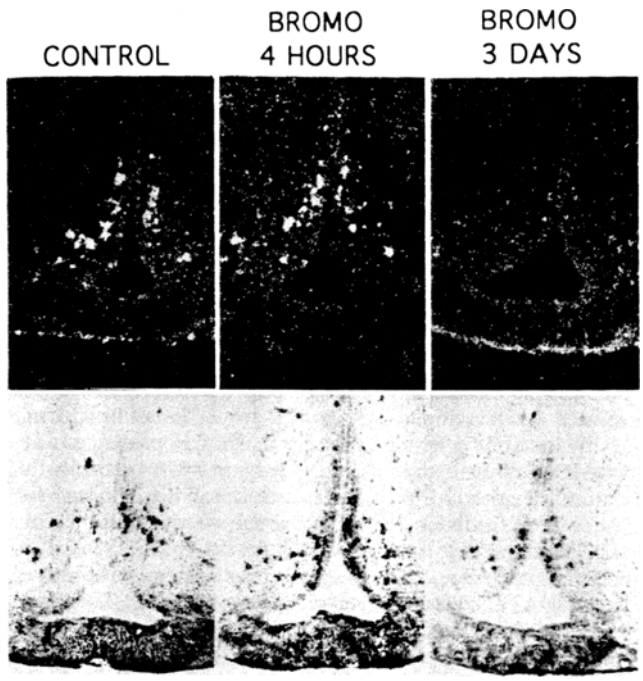

Figure 2 Tyrosine hydroxylase mRNA-containing (top panel) and tyrosine hydroxylase immunoreactive (bottom cells) in the hypothalamus after acute (4 h) or chronic (3 days) BROMO treatment of diestrous rats. Darkfield and lightfield photomicrographs show coronal sections through the arcuate nucleus at the level of the mid-median eminence. Note the presence of cell bodies in the arcuate nucleus with a dense accumulation of silver grains. The density of silver grains in tyrosine hydroxylase mRNA-containing cells in the arcuate nucleus was not altered 4 h after BROMO treatment, but was markedly reduced after 3 days. The intensity of tyrosine hydroxylase immunostaining in the arcuate-median eminence region was similar to control after 4 h of BROMO treatment, but was diminished after 3 days of BROMO treatment

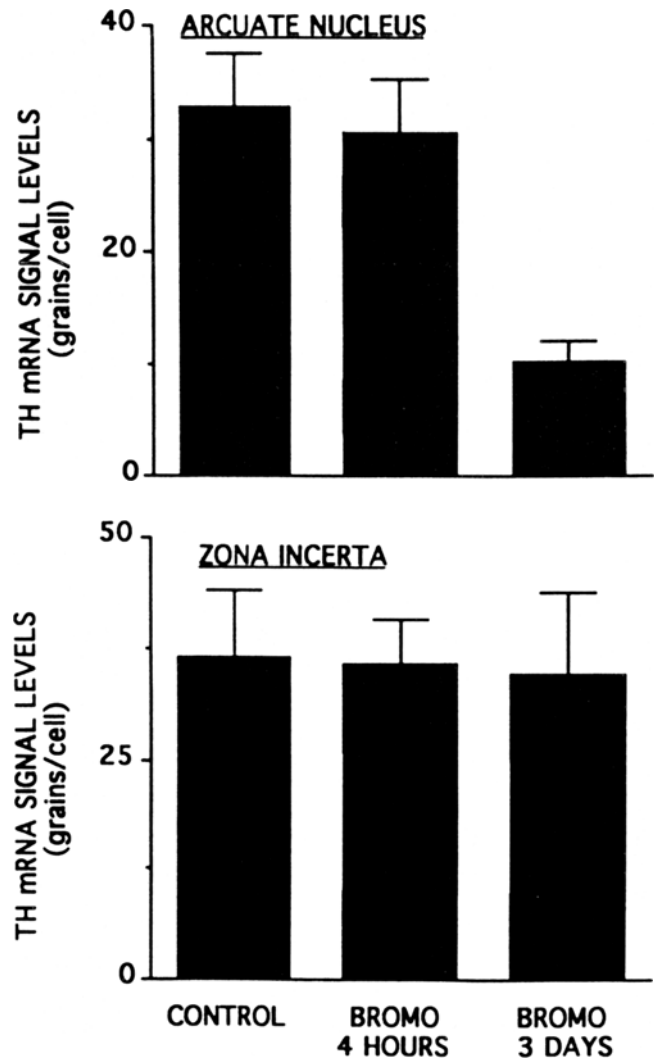

Figure 3 The relative tyrosine hydroxylase mRNA signal levels in the arcuate nucleus (top panel) and the zona incerta (bottom panel) after acute (4 h) or chronic (3 days) BROMO treatment. Cellular tyrosine hydroxylase mRNA signal levels in the arcuate nucleus were not altered 4 h after BROMO treatment, but were reduced ($P<0.05$) after 3 days of BROMO treatment. Tyrosine hydroxylase mRNA signal levels were not altered in the zona incerta after BROMO treatment. Each value is a mean ± SE of determinations from 6 rats

oPRL administration alone did not increase enzyme activity above controls. Circulating levels of endogenous rPRL were markedly suppressed by BROMO and/or oPRL treatment (Figure 4; bottom panel). Plasma rPRL levels were elevated ($P<0.05$) 15-fold when exogenous rPRL was injected with BROMO.

Discussion

The degree of PRL input is critical in determining the activity of the tuberoinfundibular dopaminergic neurons in the hypothalamus (Moore, 1987). However, it is not clear what intracellular mechanism(s) are involved in the response to PRL. Catecholaminergic neurons can respond to hormonal or neuronal inputs by rapid, transient changes in the phosphorylation state of tyrosine hydroxylase or by long-term alterations in the synthesis/degradation of the enzyme protein (Masserano & Weiner, 1983). The data in this study suggest that protein dephosphorylation contributes to the acute decrease in tyrosine hydroxylase activity caused by the suppression of PRL, whereas a reduction in tyrosine hydroxylase mRNA and protein levels plays a role in the chronic effect of suppressed PRL. Thus, enzyme inactivation may be the initial event followed by suppression of tyrosine hydroxylase gene expression. This sequence of events appears to be similar in diestrous and ovariectomized rats, and thus may be a common order induced by hypoprolactinemia, not unique to a specific endocrine condition.

Tyrosine hydroxylase activity in the SME was reduced 4 h after bromocriptine treatment coincident with suppressed circulating PRL levels. Co-administration of oPRL or rPRL prevented the effect of BROMO. These data are in general agreement with the previous report by Demarest *et al.* (1984) that BROMO significantly reduced DOPA accumulation in the median eminence within 4 h, and PRL reversed the decrease after 24 h of BROMO treatment. In the present study, okadaic acid, a phosphoprotein phosphatase 1 and 2A inhibitor (Biolojan & Takai, 1988) was used to assess whether blocking dephosphorylation could reverse the BROMO-induced decrease in tyrosine hydroxylase activity in the SME. After acute (4 h) BROMO treatment, okadaic acid increased tyrosine hydroxylase activity in the SME to levels similar to that observed in the SME of control diestrous or ovariectomized rats. In spite of the marked decrease in enzyme activity after acute BROMO treatment, tyrosine hydroxylase mRNA levels in the arcuate nucleus and tyrosine hydroxylase immunostaining in the arcuate-median eminence were not altered. Thus, the decrease in enzyme activity without any apparent change in the amount of tyrosine hydroxylase protein would indicate that alterations in the synthesis/degradation of tyrosine hydroxylase do not contribute to the short-term effect of bromocriptine. Taken together, these data suggest that protein dephosphorylation may be the

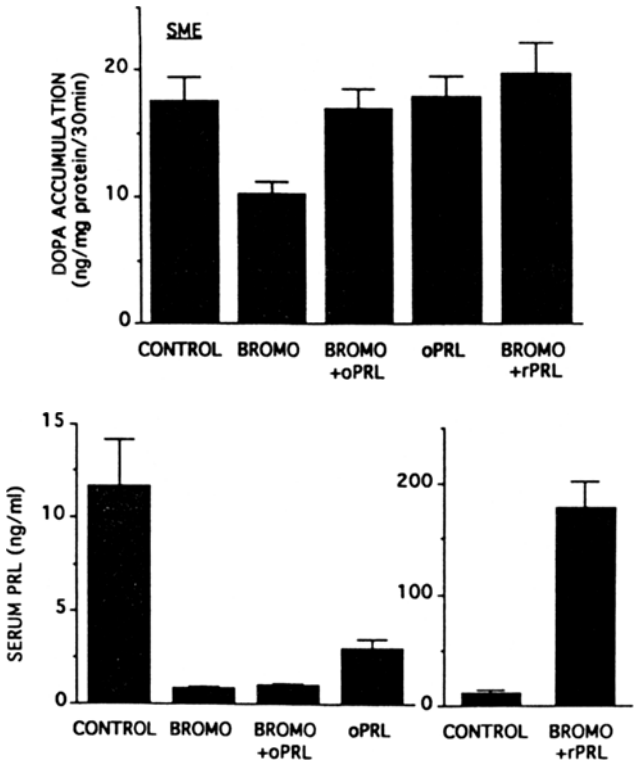

Figure 4 *In vitro* DOPA accumulation in the SME (top panel) and circulating PRL levels (bottom panel) in diestrous rats after acute (4 h) BROMO treatment. Rats were injected with oPRL or rPRL as indicated. *In vitro* DOPA accumulation was reduced ($P<0.05$) 4 h after BROMO treatment. The BROMO-induced decrease was reversed ($P<0.05$) by co-administration of oPRL or rPRL, whereas oPRL by itself had no effect. Circulating concentrations of endogenous rPRL were reduced ($P<0.05$) by BROMO and oPRL treatment, whereas rPRL injections were effective in increasing ($P<0.05$) circulating rPRL levels. Each value is a mean ± SE of determinations from 10–11 rats

primary mechanism for the rapid BROMO-dependent suppression in tyrosine hydroxylase activity. It is not clear from this study if okadaic acid increased tyrosine hydroxylase activity by augmenting the phosphate incorporation into tyrosine hydroxylase molecules or if other phosphoproteins are involved. However, it is likely that the changes in catalytic activity are caused by alterations in the phosphorylation state of tyrosine hydroxylase since this enzyme contains multiple phosphorylation sites and okadaic acid increases the phosphate incorporation of tyrosine hydroxylase in adrenal chromaffin cells and striatal synaptosomes (Haavik *et al.*, 1989).

In contrast to the striking effect of okadaic acid after acute BROMO, the response to okadaic acid was blunted after chronic (3 days) BROMO treatment, indicating that protein dephosphorylation contributes less to the reduction in catalytic activity after sustained suppression of PRL. Furthermore, tyrosine hydroxylase mRNA levels in the arcuate nucleus were markedly reduced after chronic BROMO, indicating that a decrease in the expression of the tyrosine hydroxylase gene may contribute functionally to the lower tyrosine hydroxylase activity during long-term hypoprolactinemia. Demarest *et al.* (1984) reported that tuberoinfundibular dopaminergic neurons respond to a single injection of PRL, 24 h after initiation of BROMO treatment, but that the neuronal responsiveness to PRL is diminished after chronic BROMO treatment (Demarest *et al.*, 1985). A decrease in tyrosine hydroxylase mRNA levels and enzyme quantity in the arcuate nucleus/median eminence may contribute to the inability of the tuberoinfundibular dopaminergic neurons to respond to acute PRL stimulation. However, the chronic BROMO-induced decrease in tyrosine hydroxylase activity in the SME and mRNA levels in the arcuate nucleus was prevented when elevated PRL concentrations were continuously maintained by co-administration of oPRL (Arbogast & Voogt, 1991).

Dopamine neurons from the nigrostriatal and tuberoinfundibular dopaminergic neurons have different characteristics and regulation. The dopamine neurons with nerve terminals in the SME appear to lack dopamine autoreceptors, whereas those neurons with terminals in the striatum have autoreceptors (Kehr *et al.*, 1972; Demarest & Moore, 1979; Galloway *et al.*, 1986). The BROMO-induced decrease in tyrosine hydroxylase activity in the SME is reversed by co-administration of PRL at 4 h (this study) and 3 days (Arbogast & Voogt, 1991), suggesting that the effect is not mediated by the dopamine agonist properties of this drug, but by its ability to suppress PRL. In the present study, BROMO treatment did not alter tyrosine hydroxylase in the striatum at either 4 h or 3 days, in spite of the evidence for autoreceptor feedback in this neuronal system (Kehr *et al.*, 1972; Demarest & Moore, 1979; Galloway *et al.*, 1986). It is likely that the selected times after drug administration may account for a lack of effect. Dopamine agonists decrease tyrosine hydroxylase activity in the striatum at 30–60 min (Kehr *et al.*, 1972; Demarest & Moore, 1979; Galloway *et al.*, 1986). A 4 h time period was chosen in the present study since Demarest *et al.* (1984) observed an significant decrease in enzyme activity in the SME at 4 h, but not 2 h. In contrast to the differential response to okadaic acid in the SME after acute and chronic treatment, BROMO did not alter the ability of okadaic acid to increase tyrosine hydroxylase activity in the striatum at either time. These results are in agreement that tyrosine hydroxylase mRNA levels in the substantia nigra were not altered after 3 days of BROMO treatment (Arbogast & Voogt, 1991).

In conclusion, the data in this study support the notion that a decline in circulating PRL for a short period results in decreased tuberoinfundibular dopaminergic neuronal activity via a short-term mechanism, possibly phosphorylation of tyrosine hydroxylase. This study extends previous observations that the long term absence of PRL may compromise the functional integrity or normal development of these neurons (Demarest *et al.*, 1985; Shyr *et al.*, 1986; Morgan & King, 1986; Arbogast & Voogt, 1991; Romero & Phelps, 1993). It appears that the absence of PRL initiates an integrated response within the neurons. However, it is not clear how the early event(s) (protein dephosphorylation), relates to the later event(s) (suppressed gene expression) and whether the same intracellular signals contribute to all events in the neuron.

Materials and methods

Animals

Female (200–250 g) Sprague-Dawley rats were obtained from Sasco (Omaha, NE) and housed under controlled temperature (22°C) and lighting (lights on 0600–1800 h) Rats were supplied with food and water *ad libitum*. The estrous cycle of some rats were followed by daily vaginal lavage and rats displaying at least two consecutive 4 day estrous cycles were used on diestrous day 2. Other rats were ovariectomized under ether anesthesia two weeks before use. The following treatment groups were used: (1) Control: rats were injected subcutaneously (s.c.) with the vehicle solution for BROMO (0.4% tartaric acid, 30% ethanol) and/or with the vehicle solution for PRL (0.1 M sodium bicarbonate in saline, pH 8.8) either 4 h before sacrifice or at 12 h intervals for 3 days; (2) BROMO–4 h: rats were injected with bromocriptine mesylate (3 mg/kg; s.c.) 4 h before sacrifice; (3) BROMO–3 Days: rats were injected with bromocriptine mesylate (3 mg/kg; s.c.) at 12 h intervals (1100 and 2300 h)

for 3 days and rats were sacrificed 4 h after the final BROMO injection; (4) BROMO + oPRL–4 h: rats were injected with bromocriptine mesylate (3 mg/kg; s.c.) 4 h before sacrifice and with oPRL. (NIDDK oPRL 19; 0.5 mg/kg; s.c.) or rPRL (NIDDK rPRL B-6; 0.5 mg/kg; s.c.) at 4 h and 2 h before sacrifice; (5) oPRL–4 h: rats were injected with oPRL (0.5 mg/kg; s.c.) at 4 h and 2 h before sacrifice.

Estimation of tyrosine hydroxylase activity

Tyrosine hydroxylase activity was estimated by the *in vitro* accumulation of DOPA in the SME and striatum as described previously (Arbogast & Voogt, 1991). The medial basal hypothalamus was preincubated under 95% O_2–5% CO_2 for 15 min at 37°C in 300 µl Earle's balanced salt solution containing 20 µM tyrosine. The preincubation medium was removed and 200 µl medium containing 100 µM brocresine (4-bromo-3-hydroxybenyzloxyamine: gift from American Cyanamid Co., Pearl River, N.Y., USA), an L-aromatic amino acid decarboxylase inhibitor (Chalfie & Perlman, 1977) was added. Where indicated, the incubation medium contained either 2 µM okadaic acid (Wako Chemicals, Richmond, VA, USA), a protein phosphatase 1 and 2A inhibitor (Biolojan & Tokai, 1988), or its vehicle (0.25% dimethylsulfoxide). After a 30 min period the SME was dissected with a pair of fine scissors. The SME was homogenized in 60 µl 0.1 n perchloric acid and centrifuged at 10 000 *g* for 2 min. The tissue content of L-DOPA in the supernatant was determined by HPLC with electrochemical detection as described previously. The pellet was solubilized in 0.5 N sodium hydroxide and analysed for protein content by the method of Bradford (1976). The protein content of the SME was 20–30 µg protein/SME.

Tissue collection for in situ *hybridization or immunocytochemistry*

Brains were removed rapidly after decapitation, frozen in liquid Freon (−70°C) and stored at −70°C until sectioning. Coronal sections (15 µm) were cut through the arcuate nucleus at −20°C and thaw mounted on poly-L-lysine coated slides. Alternate sections were used for *in situ* hybridization to assess tyrosine hydroxylase mRNA content or for immunocytochemistry to evaluate tyrosine hydroxylase protein. Slides were stored at −70°C. The brain sections were fixed with 4% paraformaldehyde on the assay day.

Determination of tyrosine hydroxylase mRNA by in situ *hybridization*

After the prehybridization steps described previously (Arbogast & Voogt, 1991; Voogt *et al.*, 1990), the fixed brain sections were hybridized for 4 h at 45°C with 0.05 µg/ml of ^{35}S-labeled cRNA probe for tyrosine hydroxylase. The probe was 1.1-kb Bam HI/Eco RI insert subcloned into a pSP65 vector (Promega Biotech, Madison, WI). This probe has previously been shown to hybridize to a single mRNA band of approximately 1.9 kb on Northern blots (Voogt *et al.*, 1990). A single-stranded ^{35}S-labeled antisense RNA probe complementary to tyrosine hydroxylase mRNA was synthesized using a SP6 RNA polymerase. The probe had specific activities of about 2×10^9 dpm/µg. Following RNAase treatment and a series of post hybridization washes that increased in stringency, the slides were dipped in Ilford Emulsion (K-5), diluted 0.25 g/ml water. The autoradiographs were exposed at 4°C for 4 weeks. After development using standard photographic techniques, the sections were poststained lightly with hematoxylin.

The anatomical locations of the tissue sections were determined using the rat brain atlas of Paxinos and Watson (Paxinos & Watson, 1986). Twenty alternate sections per animal through the arcuate nucleus were used for quantitation of mRNA signal levels. The rostral border was −2.5 mm and the caudal border −3.1 mm from the bregma. Approximately 18 cells/tissue section (i.e. 360 cells/rat) were analysed. Tyrosine hydroxylase mRNA-containing cells were identified under darkfield optics as a cluster of reduced silver grains with an identifiable cell nucleus. The number of grains in individual tyrosine hydroxylase mRNA-containing cells were measured under 400 × darkfield illumination by a computerized image-processing system (Georgia Instruments, Roswell, GA).

Immunocytochemistry for tyrosine hydroxylase protein

The method for tyrosine hydroxylase immunocytochemistry has been described previously (Arbogast & Voogt, 1991). The sections from various experimental groups were processed simultaneously. Briefly, after fixing the tissue, the endogenous peroxidase activity was quenched using 0.6% hydrogen peroxide in methanol. The sections were then incubated for 20 h at room temperature with a rabbit antibody to rat tyrosine hydroxylase (East Acres Biologicals, Southbridge, MA) diluted 1:2600. Brain sections were incubated successively with biotinylated goat anti-rabbit immunoglobulin G for 30 min and with Avidin DH-biotinylated horseradish peroxidase H complex (Vector Laboratories, Burlingame, CA) for 60 min. The slides were reacted for 5 min with 3.3'-diaminobenzidine (0.6 mg/ml) and 0.3% hydrogen peroxide for the peroxidase reaction.

PRL determinations

Serum PRL concentrations were determined by rat PRL RIA kit provided by NIDDK. PRL RP-3 was used as a reference preparation and the limit of sensitivity for the assay was 50 pg. The intra- and inter-assay coefficients of variation were 12.1 and 9.1%, respectively.

Statistical analysis

Results are expressed as means ± SE. The n for all groups refers to the number of experimental animals. For cellular tyrosine hydroxylase mRNA signal level determinations, the mean number of grains per cell in a given anatomical area was first calculated for individual animals. The individual means were used to calculate the mean ± SE of each group. Data were evaluated by analysis of variance and multiple comparisons were made with Fisher's least significant procedures (Zar, 1984; Gerald, 1990). Immunocytochemistry for tyrosine hydroxylase was evaluated qualitatively.

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