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Calmodulin and a Cyclic Nucleotide-Dependent Protein Kinase Facilitate the Prolactin-Induced Increase in Tyrosine Hydroxylase Activity in Tuberoinfundibular Dopaminergic Neurons

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Many aspects of tuberoinfundibular dopaminergic neuronal function are increased by elevated prolactin (PRL) levels, including the activity of tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of dopamine. This study evaluated the roles of calmodulin, cyclic nucleotide-dependent protein kinase, and calcium/calmodulin-dependent protein kinase II in the PRL-induced increase in tyrosine hydroxylase activity. Ovariectomized rats were treated with haloperidol or ovine PRL (oPRL) for 20–30 h before the experiment, respectively. Treatment with haloperidol increased circulating PRL levels 8-fold and tyrosine hydroxylase activity in the stalk-median eminence 1.8-fold. Treatment with oPRL increased tyrosine hydroxylase activity 1.9-fold. W-7, a calmodulin antagonist, reversed both the haloperidol- and oPRL-induced increase in tyrosine hydroxylase activity to control levels. H-8, a cyclic nucleotide-dependent protein kinase inhibitor, also reversed the haloperidol induced increase in tyrosine hydroxylase activity. KN62, a selective calcium/calmodulin-dependent protein kinase II inhibitor, attenuated the haloperidol-induced increase in tyrosine hydroxylase activity, but KNO4, a structurally related control compound, had no effect. By contrast, the oPRL- and haloperidol-induced increases in tyrosine hydroxylase activity were not altered by KN93, a selective calcium/calmodulin-dependent protein kinase II inhibitor. These data indicate that calmodulin and a cyclic nucleotide-dependent protein kinase contribute to the PRL-induced increase in tyrosine hydroxylase activity, but the role of calcium/calmodulin-dependent protein kinase II is still unclear.

Key Words: Tyrosine hydroxylase; prolactin; cyclic nucleotide; calmodulin.

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Introduction

Dopamine released from the tuberoinfundibular dopaminergic (TIDA) neurons in the hypothalamus is the major prolactin (PRL) release–inhibiting hormone *(1)*. PRL, in turn, exerts a negative feedback to increase the secretion, synthesis, and metabolism of dopamine *(2–7)*. Although this feedback loop is well established, the cellular signals, which mediate the increase in TIDA neuronal activity, are not completely understood. PRL receptors are localized on TIDA neurons, providing a histologic basis for a direct action of PRL on these neurons *(8,9)*. The exact role of this direct effect in controlling specific aspects of neuronal cell function has not yet been clearly defined. However, other cells also contain PRL receptors and an indirect action mediated by separate neuronal input cannot be ruled out. Indeed, Hentschel et al. *(10)* report that blocking neurotensin receptors with a selective antagonist reverses the hyperprolactinemia-induced increase in TIDA neuronal activity. Taken together, these studies suggest that the TIDA neurons may integrate multiple signals for the appropriate cellular response to PRL feedback.

Tyrosine hydroxylase is the initial and rate-limiting enzyme in the catecholaminergic biosynthetic pathway *(11)*. The activity of this enzyme can be regulated by short-term activation of the existing enzyme or by modulation of the amount of enzyme *(12–14)*. The immense complexity of this regulation is a reflection of its key position in the synthesis of neurotransmitters and neurohormones in both the central and peripheral nervous systems. Tyrosine hydroxylase is a substrate for multiple protein kinases. The N-terminal domain of tyrosine hydroxylase contains four serines (Ser^{8}, Ser^{19}, Ser^{31}, Ser^{40}), which serve as regulatory phosphorylation sites *(13–16)*. Cyclic adenosine monophosphate (cAMP)-dependent protein kinase phosphorylates Ser^{40} *(16)*, whereas calcium/calmodulin-dependent protein kinase II phosphorylates primarily Ser^{19}, but also phosphorylates Ser^{40} under some conditions *(16,17)*. Ser^{31} is a substrate for extracellular signal-regulated kinases (ERKs), ERK 1 and ERK 2 *(18)*. In addition to acute regulatory mechanisms, molecular regulation of tyrosine hydroxylase is also complex, involving transcriptional regulation of the tyrosine

hydroxylase gene by multiple transcription factors, modulation of mRNA stability, and translational regulation *(14)*.

PRL alters both tyrosine hydroxylase gene expression and protein phosphorylation in the TIDA neurons *(3,4,19)*, indicating multiple cellular levels of regulation for this enzyme by PRL feedback. The timing, level of circulating PRL, or method of creating hyperprolactinemia may influence the cellular mechanisms that regulate tyrosine hydroxylase activity in the TIDA neurons. For example, although hyperprolactinemic conditions in general increase tyrosine hydroxylase activity, treatment with ovine PRL (oPRL), but not haloperidol, increases tyrosine hydroxylase gene expression *(3)*. However, immunoneutralization studies indicate that the effect of haloperidol on TIDA neuronal activity is mediated by the haloperidol-induced elevation in circulating PRL *(20)*. Protein phosphorylation appears to be important in maintaining appropriate basal levels of tyrosine hydroxylase activity, as evidenced by the ability of okadaic acid to reverse the bromocriptine-induced decrease in tyrosine hydroxylase activity in the stalk-median eminence (SME) *(19)*. Protein kinase C (PKC) is involved in the initial increase in tyrosine hydroxylase activity within 2 h of in vitro oPRL treatment of hypothalamic slices *(21)*. Arita and Kimura *(22)* report calcium dependence and an enhanced response to depolarizing stimuli during haloperidol-induced hyperprolactinemia after 42 h of treatment. The objectives of the present study were to examine the involvement of calmodulin in the activation of tyrosine hydroxylase activity in TIDA neurons during hyperprolactinemia, to determine the role of cyclic nucleotide-dependent protein kinase(s) in the haloperidol-induced elevation of tyrosine hydroxylase activity in TIDA neurons, and to evaluate the contribution of calcium/calmodulin-dependent protein kinase II in elevating tyrosine hydroxylase activity in the TIDA neurons of haloperidol- or oPRL-treated rats.

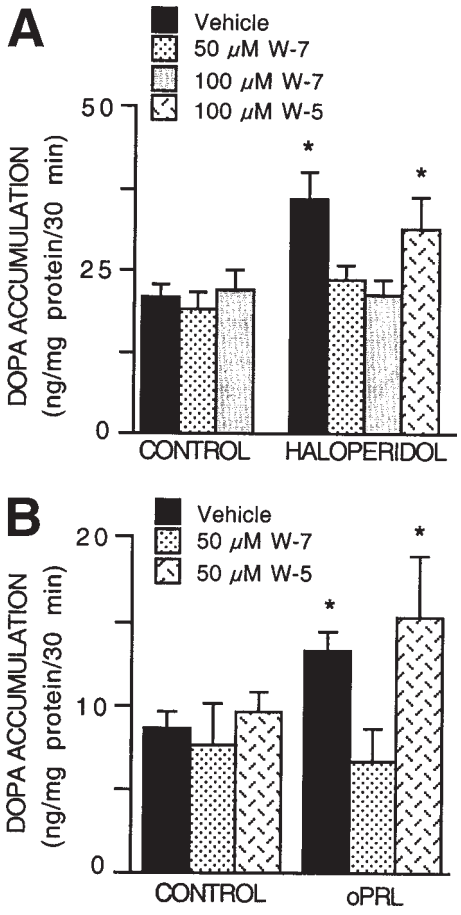

Fig. 1. Tyrosine hydroxylase activity in SME of control or haloperidol-treated rats (**A**) and of control or oPRL-treated rats (**B**) after in vitro incubation of MBH with W-7 (calmodulin antagonist), W-5 (a weaker calmodulin antagonist used as a control), or vehicle. The haloperidol- and oPRL-induced increases were reversed by W-7, but not W-5. Each value is a mean ± SE of determinations from 6 to 10 rats. *Significantly different from control-vehicle group.

Results

Effect of Calmodulin Antagonist on PRL-Induced Increase in Tyrosine Hydroxylase Activity in SME

The first experiments examined the role of calmodulin in the hyperprolactinemia-induced increase in tyrosine hydroxylase activity in the SME. Ovariectomized (OVX) rats were treated in vivo with haloperidol or oPRL for 20–30 h, respectively. Treatment with haloperidol increased circulating PRL levels from 3.2 ± 8 (n = 8) to 61.2 ± 10.1 ng/mL (n = 9) for the in vitro vehicle-treated groups. Similar increases in circulating PRL levels were observed in rats used for all other in vitro treatments as well. The means for basal PRL levels ranged from 2.3 ± 0.5 to 5.4 ± 2.1 ng/mL among the different in vitro treatment groups, whereas the range for haloperidol-induced PRL levels was 59.5 ± 5.0 to 71.8 ± 7.5 ng/mL.

In vitro L-dihydroxyphenylalanine (DOPA) accumulation was 1.8-fold greater in the SME from haloperidol-treated rats as compared with control rats treated with vehicle for haloperidol (Fig. 1A). When 50–100 μM W-7, a calmodulin antagonist *(23)*, was included in the preincubation and incubation media, the stimulatory effect of haloperidol on tyrosine hydroxylase activity was reversed, whereas W-7 had no effect on basal tyrosine hydroxylase activity in the SME of control rats. In contrast to the action of W-7, 100 μM W-5, which acts as a weaker calmodulin antagonist *(23)*, did not alter the haloperidol-induced increase in tyrosine hydroxylase activity, supporting the notion that the inhibitory action is owing to the calmodulin antagonist activity of W-7. In a similar manner, treatment with oPRL increased tyrosine hydroxylase activity in the SME 1.6-fold, and W-7, but not W-5, prevented this stimulatory effect (Fig. 1B).

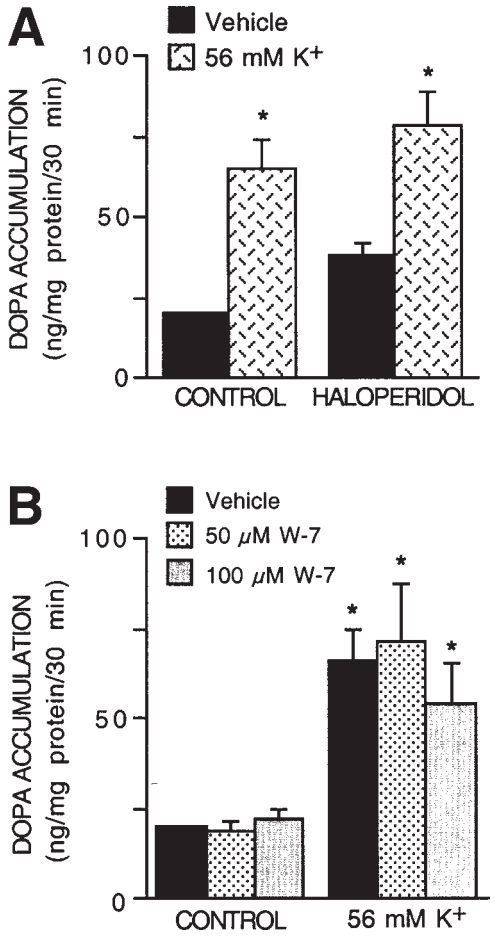

Fig. 2. (**A**) Tyrosine hydroxylase activity in SME of control and haloperidol-treated rats after in vitro incubation of MBH with or without 56 m*M* K^+. (**B**) Tyrosine hydroxylase activity in SME of untreated OVX rats after in vitro incubation of MBH with or without 56 m*M* K^+ and W-7 (calmodulin antagonist) or vehicle. W-7 treatment did not significantly alter the K^+-induced increase in tyrosine hydroxylase activity. Each value is a mean ± SE of determinations from 8 to 10 rats. *Significantly different from control-vehicle group.

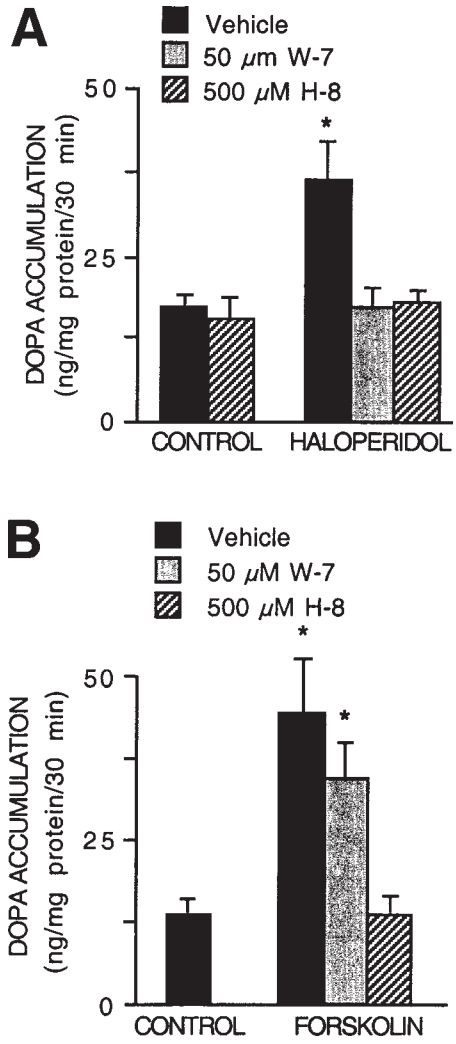

Fig. 3. (**A**) Tyrosine hydroxylase activity in SME of control and haloperidol-treated rats after in vitro incubation of MBH with W-7 (a calmodulin antagonist), H-8 (a cyclic nucleotide-dependent protein kinase inhibitor), or vehicle. (**B**) Tyrosine hydroxylase activity in SME of untreated OVX rats after in vitro incubation of MBH with W-7 (calmodulin antagonist), H-8 (cyclic nucleotide-dependent protein kinase inhibitor), or vehicle. Both W-7 and H-8 reversed the stimulatory effect of haloperidol. The forskolin-induced increase in tyrosine hydroxylase activity was prevented by H-8, but not W-7. Each value is a mean ± SE of determinations from 7 to 10 rats.

The next experiment examined interactions between in vivo haloperidol treatment and in vitro K^+-induced depolarization on tyrosine hydroxylase activity in the SME (Fig. 2A). In vivo haloperidol treatment caused a 1.8-fold increase in tyrosine hydroxylase activity in the SME, whereas in vitro incubation of MBH with 56 m*M* K^+ resulted in a 3.2-fold increase in tyrosine hydroxylase activity. The incubation of mediobasal hypothalamus (MBH) fragments from haloperidol-treated rats with 56 m*M* K^+ resulted in a 3.9-fold increase.

The role of calmodulin in the depolarization-induced increase in tyrosine hydroxylase activity was examined in the SME of untreated OVX rats (Fig. 2B). In vitro incubation of MBH fragments with 56 m*M* K^+ increased tyrosine hydroxylase activity in the SME 3.2-fold. Coincubation with W-7 did not alter basal tyrosine hydroxylase activity or the depolarization-induced increase in tyrosine hydroxylase activity.

Effect of a Cyclic Nucleotide-Dependent Protein Kinase Inhibitor on PRL-Induced Increase in Tyrosine Hydroxylase Activity

We examined the role of cyclic nucleotide-dependent protein kinase(s) in the PRL-induced increase in tyrosine hydroxylase activity in the SME (Fig. 3A). In vivo haloperidol treatment increased in vitro DOPA accumulation in the SME 2.1-fold. The haloperidol-induced increase was abolished when either 50 μ*M* W-7, a calmodulin antagonist,

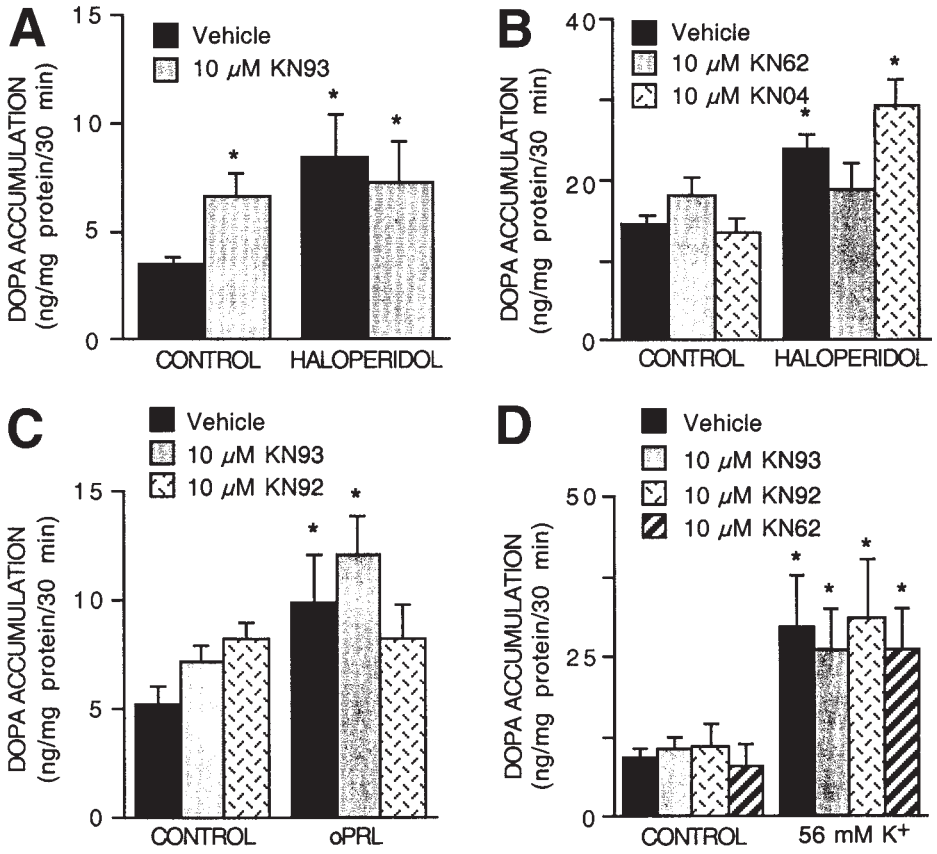

Fig. 4. Tyrosine hydroxylase activity in SME of control and haloperidol-treated rats after in vitro incubation of MBH with (**A**) KN93 (calcium/calmodulin-dependent protein kinase II inhibitor) or (**B**) KN62 (calcium/calmodulin-dependent protein kinase II inhibitor), KN04 (structurally related compound used as a control) or vehicle. (**C**) Tyrosine hydroxylase activity in SME of control and oPRL-treated rats after in vitro incubation of MBH with KN93, KN92 (structurally related compound used as a control), or vehicle. (**D**) Tyrosine hydroxylase activity in SME of untreated OVX rats after in vitro incubation of MBH with or without 56 m*M* K^+ and KN93, KN92, KN62, or vehicle. KN62 reversed the haloperidol-induced increase in tyrosine hydroxylase activity in the SME, whereas KN93 did not alter the haloperidol- or oPRL-induced increase. Calcium/calmodulin-dependent protein kinase II inhibitors did not affect the increase in tyrosine hydroxylase activity owing to K^+ depolarization. Each value is a mean ± SE of determinations from six to eight rats. *Significantly different from control-vehicle group.

or 500 μ*M* H-8, a cyclic nucleotide-dependent protein kinase inhibitor *(24)*, was included in the preincubation and incubation media. H-8 had no effect on basal tyrosine hydroxylase activity in the SME from control rats.

To evaluate the ability of W-7 and H-8 to inhibit a cAMP-dependent response, MBH fragments from untreated OVX rats were incubated with 1 μ*M* forskolin, an activator of adenylate cyclase (Fig. 3B). Forskolin increased tyrosine hydroxylase activity in the SME threefold. The stimulatory effect of forskolin was prevented by preincubation and incubation with H-8, but not W-7.

Effect of Calcium/Calmodulin-Dependent Protein Kinase Inhibitor on PRL-Induced Increase in Tyrosine Hydroxylase Activity

The last set of experiments evaluated the role of calcium/calmodulin-dependent protein kinase II in the PRL-induced increase in tyrosine hydroxylase activity by using selective inhibitors (Fig. 4). Tyrosine hydroxylase activity in the SME of haloperidol-treated rats was 1.5- to 2.4-fold greater than that of vehicle-treated control rats (Fig. 4A,B). After in vitro incubation with KN62, a selective calcium/calmodulin-dependent protein kinase II inhibitor *(25)*, tyrosine hydroxylase activity was similar in the SME of haloperidol-treated and control rats, suggesting that KN62 prevented the haloperidol-induced increase in tyrosine hydroxylase activity (Fig. 4B). KN04, the control analog, did not alter the haloperidol-induced increase in tyrosine hydroxylase activity. In contrast to KN62, in vitro treatment with KN93, another calcium/calmodulin-dependent protein kinase II inhibitor *(26)*, did not alter the haloperidol-induced increase in tyrosine hydroxylase activity but did significantly increase tyrosine hydroxylase activity in the SME of control rats (Fig. 4A). In a similar manner, tyrosine hydroxylase activity in the SME from oPRL-treated rats was similar after in vitro treatment with KN93 or its vehicle, respectively

(Fig. 4C). Tyrosine hydroxylase activity in the SME of oPRL-treated rats was similar to control-vehicle levels after in vitro treatment with KN92, the control analog. K^+-induced depolarization caused a 3.2-fold increase in tyrosine hydroxylase activity in the SME of untreated OVX rats, and KN93, KN92, and KN62 did not alter the depolarization-induced increase (Fig. 4D).

Discussion

Calmodulin and a cyclic nucleotide-dependent protein kinase(s) facilitated the PRL-induced increase in tyrosine hydroxylase activity in the SME. W-7, a calmodulin antagonist reversed the increase in tyrosine hydroxylase activity with hyperprolactinemia caused by treatment with haloperidol or oPRL. H-8, a cyclic nucleotide-dependent protein kinase inhibitor, reversed the increase in tyrosine hydroxylase activity under conditions of haloperidol-induced hyperprolactinemia. Calcium/calmodulin-dependent protein kinase II is a target for calmodulin regulation in the presence of calcium, but the role of this protein kinase in modulating the effects of PRL on dopamine biosynthesis in the TIDA neurons is still unclear. KN62, a selective calcium/calmodulin-dependent protein kinase inhibitor, partially reversed the haloperidol-induced increase in tyrosine hydroxylase activity, whereas KN93 did not alter the PRL-induced increase in tyrosine hydroxylase activity.

PRL's effect on the TIDA neurons has a rapid "tonic" component as well as a delayed "induction" component *(6,27)*. Thus, it is likely that the present study examined cellular changes, which contribute to the stimulatory action of PRL during the induction component. Moreover, these cellular changes may differ from those during the early tonic component. These data implicate that calmodulin contributes to the sustained action of PRL on TIDA neurons. The ability of W-7 to reverse completely the stimulatory effects of both haloperidol and oPRL treatment supports the notion that calmodulin is involved in the hyperprolactinemia-induced increase in tyrosine hydroxylase activity. The fact that W-5, the dechlorinated control analog, had no effect on the hyperprolactinemia-induced increase in tyrosine hydroxylase activity provides specificity that the blocking action was owing to W-7's ability to antagonize calmodulin binding, rather than nonspecific actions.

Although calmodulin was implicated in the response of the TIDA neurons to hyperprolactinemic conditions, the involvement of calcium/calmodulin-dependent protein kinase II was not clearly identified in the present study. After calcium binding, calmodulin plays an important role in controlling neuronal function, and calcium/calmodulin-dependent protein kinase II is one important target protein. This protein kinase regulates tyrosine hydroxylase activity by increasing the phosphorylation of Ser^{19} and Ser^{40} *(16,17)*, although an activator protein is required for an increase in tyrosine hydroxylase catalytic activity *(28)*. Kinetic analysis indicates that enzyme activation is associated with an increase in V_{max} *(28)*. Likewise, an increase in V_{max} for tyrosine hydroxylase in the TIDA neurons is the kinetic change observed after 4 d of haloperidol treatment *(29)*. Thus, a reasonable hypothesis was that tyrosine hydroxylase activation by haloperidol treatment might involve calcium/calmodulin-dependent protein kinase II. In the presence of a selective calcium/ calmodulin-dependent protein kinase II inhibitor, tyrosine hydroxylase activity in the SME of haloperidol-treated rats was similar to enzyme activity in the SME of control rats after in vitro treatment with vehicle or KN62. These data would implicate some involvement of this protein kinase in the haloperidol-induced increase in dopamine synthesis. By contrast, KN93, a selective calcium/calmodulin-dependent protein kinase II inhibitor, had no effect on the oPRL- or haloperidol-induced increase in tyrosine hydroxylase activity. An increase in the amount of enzyme protein is another potential mechanism to increase the V_{max} for an enzyme. Indeed, tyrosine hydroxylase gene expression is increased after oPRL treatment, but tyrosine hydroxylase mRNA signal levels are not altered after 3 d of haloperidol treatment *(3)*. The rise in PRL appears to mediate the haloperidol-induced increase in TIDA neuronal activity as evidenced by immunoneutralization studies *(20)*. Our data support this concept, but other cellular mechanisms may play a role. A direct dopamine antagonist action on the TIDA neurons by haloperidol cannot be entirely ruled out. Indeed, the TIDA neurons can be regulated by dopamine D2 receptors, although these receptors do not appear to be autoreceptors *(30)*.

Given the differential response to selective calcium/calmodulin-dependent protein kinase II inhibitors after oPRL and haloperidol treatment, other mechanism(s) should be considered. The involvement of other calmodulin-regulated proteins cannot entirely be ruled out. Indeed, Albarracin et al. *(31)* reported PRL regulation of calmodulin-dependent protein kinase III in the rat corpus luteum *(31)*. W-7 was first identified as a calmodulin antagonist *(32)* but has subsequently been shown to alter calcium influx in some cell types *(33–36)*. Likewise, KN-62 may influence calcium influx in some cell types independent of calcium/calmodulin-dependent protein kinase II *(37,38)*. Without clear evidence of the involvement of a documented calmodulin binding protein, some caution should be taken in interpreting the mechanism(s) for calmodulin's involvement in the hyperprolactinemia-induced increase in tyrosine hydroxylase activity. In spite of this caveat, the data in the present study support the concept that calcium is essential for PRL to increase dopamine synthesis in the TIDA neurons, even though the exact mechanism was not clearly identified. These data are in general agreement with the findings of Arita and Kimura *(22)*, who reported that the haloperidol-induced increase in tyrosine hydroxylase activity was abolished by

the addition of tetrodotoxin or the removal of calcium. Their data indicate that this increase in dopamine biosynthesis is calcium dependent and involves tetrodotoxin-sensitive sodium channels.

The haloperidol-induced increase in tyrosine hydroxylase activity in the SME was reversed by H-8, a cyclic nucleotide-dependent protein kinase inhibitor. H-8 is a potent inhibitor of both cyclic guanosine 5'-monophosphate (cGMP)- and cAMP-dependent protein kinase and acts by competing with the adenosine triphosphate binding site *(39,40)*. Activation by cAMP-dependent protein kinase is perhaps the best-characterized intracellular signaling pathway involved in the regulation of tyrosine hydroxylase. cAMP-dependent protein kinase phosphorylates Ser^{40} *(16)* and results in an increased affinity of tyrosine hydroxylase for its pterin cofactor *(41, 42)*. Although less extensively studied, cGMP-dependent protein kinase phosphorylates the same residue as cAMP-dependent protein kinase and causes similar kinetic changes *(42)*. Although they have not been identified as part of the PRL response, neuropeptides such as vasoactive intestinal peptide *(43)*, pituitary adenylate cyclase-activating polypeptide *(43,44)*, and calcitonin *(45)* increase TIDA neuronal activity. cAMP-dependent phosphorylation mediates the stimulatory effect of vasoactive intestinal peptide on tyrosine hydroxylase activity in bovine chromaffin cells *(46)*. Likewise, a cAMP-dependent mechanism is responsible for the salmon calcitonin–induced increase in tyrosine hydroxylase activity in fetal hypothalamic cells *(45)*. Although the kinetic changes observed with haloperidol treatment are inconsistent with cAMP-dependent protein kinase *(29,41,42)*, there may be interaction between calcium- and cAMP-dependent systems. The calcium-stimulated phosphorylation of Ser^{40} is reduced by specific inhibitors of protein kinase A (PKA) and PKC *(47)*.

Our study examined the effect of increasing circulating PRL in vivo for a period of 20–30 h. Thus, the mechanisms identified are probably not the initial response to PRL, but the result of subsequent alterations in TIDA cellular function. Under some circumstances, an early increase in TIDA neuronal activity is observed within 1 to 2 h after either in vivo PRL treatment of rats *(5,6,27)* or in vitro PRL treatment of hypothalamic slices *(21)*. This acute effect appears to involve a change in the phosphorylation state of tyrosine hydroxylase, which is mediated by PKC *(21)*. Pasqualini et al. *(21)* suggest PRL activation of a PKC/ERK pathway, although activation of the ERK pathway has not yet been demonstrated in the TIDA neurons. In contrast to the present study, selective inhibitors for PKA or calmodulin did not alter this acute effect of PRL *(21)*. Although several signaling molecules have been implicated in PRL's effect on target cells, activation of the Jak 2/Stat 5 pathway is considered a primary intracellular response after PRL binding to the long form of the PRL receptor *(48–52)*. Lerant et al. *(53)* recently reported an increase in Stat 5 nuclear localization signal in hypothalamic dopaminergic neurons after oPRL treatment. However, the connections between these initial signals and signals that sustain an increase in TIDA neuronal activity have not yet been identified. The initial PRL signals may directly alter the cellular function of TIDA neurons. Alternatively, PRL may activate other neuronal inputs, which influence TIDA neurons. Indeed, neurotensin input may mediate the stimulatory effect of hyperprolactinemia after 12 h of haloperidol or rat PRL treatment *(10)*. Stimulation of rat neurotensin receptors, transfected into Chinese hamster ovary cells, results in an increase in cytosolic calcium concentration *(54)*. Thus, the findings of the present study may be consistent with neurotensin input. However, it is notable that the stimulatory action of hyperprolactinemia was retained in vitro in both the present and previous studies *(3,22)*, in spite of the fact that in vivo extrahypothalamic inputs are absent for at least 1 h in vitro.

Although both W-7 and H-8 both reversed the haloperidol-induced increase in tyrosine hydroxylase activity in the SME, these two inhibitors appear to act by different mechanisms. W-7 reversed the haloperidol-induced increase in tyrosine hydroxylase activity but did not block the K^+-induced increase in depolarization. H-8 blocked the forskolin-induced increase in tyrosine hydroxylase activity, whereas W-7 did not significantly alter this cAMP-dependent increase. Therefore, it was surprising that each of these inhibitors completely blocked the haloperidol-induced rise. Taken together, these data support the idea that multiple cellular pathways controlling dopamine biosynthesis are enhanced during conditions of hyperprolactinemia. Thus, it is likely that a certain amount of redundancy exists for this important feedback system and that the intracellular mechanisms to regulate TIDA neuronal activity are complex.

Materials and Methods

Animals

Female Sprague-Dawley rats (200–250 g) were obtained from Charles River (Raleigh, NC) and housed under controlled temperature (22ºC) and lighting (lights on 7:00 AM to 9:00 PM). Rats were supplied with food and water ad libitum. The Animal Care Committee of Southern Illinois University approved all experimental protocols. Rats were OVX 2 wk before use and were sacrificed between 2:00 PM and 4:00 PM on the day of the experiment. Haloperidol injections or oPRL infusions generated hyperprolactinemia. Rats were divided into five in vivo treatment groups:

1. Group 1: OVX rats were untreated in vivo.
2. Group 2: Rats were injected subcutaneously with the vehicle for haloperidol, 0.3% tartaric acid (1 mL/kg, subcutaneously) at 20 and 4 h before the experiment.
3. Group 3: Rats were injected with haloperidol (2.5 mg/kg, subcutaneously) (Sigma, St. Louis, MO) at 20 and 4 h before the experiment.

4. Group 4: Rats were implanted with a chronic jugular cannula at 30 h before the experiment, and vehicle for oPRL (0.01 *M* sodium bicarbonate, pH 8.6; 0.15 *M* NaCl) was injected intravenously.
5. Group 5: A chronic cannula was inserted into the jugular vein at 30 h before the experiment and connected to an osmotic minipump (Alza, Palo Alto, CA) containing oPRL (1.25 mg/200 µL). This results in a flow rate of 1 µL/h and circulating PRL levels of approx 125–150 ng/mL *(3)*.

Estimation of Tyrosine Hydroxylase Activity

Tyrosine hydroxylase activity was estimated as described previously *(3)*. Medial basal hypothalamic fragments, which contained the arcuate nucleus, SME, and part of the ventromedial nucleus, were dissected with fine scissors. The fragments were preincubated under 95% O_2- 5% CO_2 for 15 min at 37ºC in 300 µL of Earle's Balanced Salt Solution containing 20 µM tyrosine. Where indicated, the medium contained 50-100 µ*M* W-7 (Sigma), 100 µ*M* W-5 (Sigma), 500 µ*M* H-8 (BIOMOL, Plymouth Meeting, PA), 10 µ*M* KN62 (Seikagaku, Tokyo, Japan, or Calbiochem-Novabiochem, San Diego, CA), 10 µ*M* KN04 (Seikagaku), 10 µM KN93 (Calbiochem-Novabiochem), 10 µ*M* KN92 (Calbiochem-Novabiochem), or vehicle (0.1% dimethylsulfoxide). The preincubation medium was removed, and incubation medium containing 100 µ*M* brocresine and the indicated activators and inhibitors was added. Activating agents included 1 µ*M* forskolin and 56 m*M* K^+ as a depolarizing agent. When 56 m*M* K^+ was included as a depolarzing agent, KCl replaced an equivalent amount of NaCl in the medium. After a 30-min incubation period, the SME was dissected with fine scissors, homogenized in 120 µL of $HClO_4$, and centrifuged at 10,000*g* for 2 min. The pellet was solubilized in 0.5 *N* NaOH and used for protein determinations. The tissue content of DOPA was determined by high-performance liquid chromatography with electrochemical detection.

PRL Determinations

Serum PRL concentrations were determined by the rat PRL radioimmunoassay (RIA) kit provided by Dr. Albert Parlow and the National Hormone and Pituitary Program. PRL RP-3 was used as a reference preparation, and the limit of sensitivity for the assay was 50 pg. The intra- and interassay coefficients of variation were 12.1 and 9.1%, respectively.

Statistical Analyses

Results are expressed as means ± SE. The *n* for all groups refers to the number of experimental animals. Data were evaluated by two-way analysis of variance followed by Fisher's least significance procedures *(55,56)*. Differences were considered significant at $p < 0.05$.

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