Neuropeptide Y Inhibits Chromaffin Cell Nicotinic Receptor-Stimulated Tyrosine Hydroxylase Activity through a Receptor-Linked G Protein-Mediated Process

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SUMMARY

Acetylcholine stimulation of bovine chromaffin cells results in increased norepinephrine and epinephrine secretion accompanied by a corresponding increase in synthesis. The addition of neuropeptide Y (NPY) to the culture medium prevents the increase in catecholamine synthesis but not secretion. Treatment of chromaffin cells with nicotine produces a concentration-dependent increase in tyrosine hydroxylase activity (IC\(_{50}\) = 1.2 \(\mu\)M) that is reduced if NPY is present during stimulation. Tyrosine hydroxylase activity decreases in a concentration-dependent fashion if increasing amounts of NPY are included in the culture medium, IC\(_{50}\) = 0.2 nm. Treatment with pertussis toxin completely prevents the effect of NPY. The rank order of potency for inhibition of tyrosine hydroxylase activity is NPY \(\approx\) [Leu\(^{31}\),Pro\(^{34}\)]NPY \(\approx\) peptide YY > NPY2-36 > NPY13-36 > NPY18-36 \(\approx\) NPY26-36 >> NPY1-30, suggesting a NPY-Y1 receptor subtype. Examination of the effect of NPY on nicotine stimulation of chromaffin cell protein phosphorylation showed that NPY produces a concentration-dependent decrease in a 60-kDa protein, IC\(_{50}\) = 6.4 nm. The effect of NPY is pertussis toxin-sensitive. The rank order of potency is [Leu\(^{31}\),Pro\(^{34}\)]NPY \(\approx\) NPY \(\approx\) NPY18-36. Immunoprecipitation confirmed the identity of the 60-kDa protein as tyrosine hydroxylase.

NPY is a potent and pervasive peptide with actions in both the central and peripheral nervous systems. NPY can increase blood pressure and food intake and decrease anxiety symptoms (1). The physiological effects of NPY are mediated by several receptor subtypes (1), four of which have been cloned (2–7). NPY receptors are members of the family of G protein-coupled receptors and affect either inhibition of cAMP accumulation, changes in Ca\(^{2+}\) influx, or possibly, changes in inositol 1,4,5-trisphosphate turnover (1, 8).

NPY has been the subject of intensive investigation to learn more about its role in various physiological functions (1). Because NPY is co-stored and co-released from the adrenal medulla with the catecholamines (9–11), chromaffin cells make a useful model for the study of NPY action. The following observations suggest that NPY plays a physiologically significant role in the function of the adrenal medulla: (a) NPY can be secreted from chromaffin cells (12, 13); (b) adrenal medulla membranes contain high affinity binding sites for \(^{125}\)I-NPY (IC\(_{50}\) = 0.27 nm) (14); and (c) NPY inhibits forskolin-stimulated cAMP accumulation (IC\(_{50}\) = 0.9 nm) in chromaffin cells through a PTX-sensitive process (14).

One of the functions ascribed to the action of NPY on chromaffin cells is the inhibition of catecholamine secretion (15). A more detailed examination of the effect of NPY on secretion revealed that NPY modifies nicotinic receptor-stimulated \(^{3}H\)NE secretion by blocking the chromaffin cell nicotinic receptor ligand-gated ion channel (16). It seems unlikely that this effect of NPY is receptor-mediated because NPY fragments: (a) are more effective than NPY, (b) exhibit low affinity (IC\(_{50}\) = 1.4 \(\mu\)M), and (c) are effective after treatment with PTX. The effect is probably more of pharmacological than physiological interest because micromolar concentrations of NPY are required and NPY concentrations in conditioned chromaffin cell media exist in nanomolar amounts (13). Thus a physiological role for NPY action on chromaffin cells remains to be determined.

Because NPY is co-stored and co-released with catecholamines and can influence second messenger levels required for a variety of chromaffin cell functions (e.g., cAMP and Ca\(^{2+}\)), it seems plausible that the peptide may act as an important regulator of chromaffin cell activity using these...
second messengers. Because catecholamine biosynthesis in chromaffin cells is one function regulated by changes in the levels of cAMP and Ca2+ (17), we propose that NPY has the capability to regulate catecholamine biosynthesis. Evidence supporting this hypothesis has been provided by previous studies demonstrating that NPY inhibits dihydroxyphenylalanine biosynthesis in rat adrenal glands (18) and pheochromocytoma cells (19).

Catecholamine biosynthesis increases primarily through alterations in tyrosine hydroxylase activity (20) in response to various second messenger changes and the activation of protein kinases (17). In bovine chromaffin cells, activation of nicotinic receptors results in increased tyrosine hydroxylase phosphorylation (21). Phosphorylation results in a decrease in the K_m for cofactor tetrahydrobiopterin and a resultant increase in the conversion of tyrosine to dihydroxyphenylalanine (17). Tyrosine hydroxylase activity could also be regulated through events that decrease tyrosine hydroxylase phosphorylation. For example, agents that decrease intracellular Ca2+ or cAMP accumulation could decrease the activity of protein kinases responsible for tyrosine hydroxylase activity. We present evidence that NPY can inhibit catecholamine biosynthesis by inhibiting cholinergic receptor-induced tyrosine hydroxylase phosphorylation and subsequent activity.

Experimental Procedures

Cell culture. Isolation and culturing of bovine adrenal chromaffin cells was performed with modifications as described previously (14). Cells were plated on 60-mm plastic dishes at a density of 3 x 10^6 cells/dish in an atmosphere of 5% CO_2 at 37°. Cells were used between 3 and 8 days after plating.

Catecholamine secretion and determination of cell content. Culture medium was removed from chromaffin cells by two washes with KRP buffer, pH 7.4, containing 154 mM NaCl, 2.2 mM CaCl_2, 1.1 mM KCl, 1.1 mM MgSO_4, 8.5 mM Na_2HPO_4, and 10 mM glucose. Chromaffin cells were then incubated in KRP at 37° for 15 min and stimulated with ACh (3 μM) for 12 min at 37°. Cell medium was removed and stabilized with 1.6 mM NaHCO_3 and 34 mM HClO_4 and stored at −20°. The remaining cells were lysed in KRP buffer containing 0.1 mM EDTA and 0.1% Triton X-100 by freeze-thawing. Extracts were stabilized as described for the medium. Serum or cell content of EPI and NE was measured by electrochemical detection after liquid chromatography (22).

Tyrosine hydroxylase assay. Cells were washed three times and preincubated with KRP buffer at 37° for 1 hr and stimulated with various agonists dissolved in prewarmed KRP buffer for 4 min. The reaction was stopped by adding 0.45 ml of buffer containing 30 mM potassium phosphate, pH 6.8, 50 mM NaF, 0.1 mM EDTA, and 0.1% Triton X-100 and freezing in dry ice/ethanol bath. After centrifugation at 13,000 x g for 5 min, supernatants were adjusted to 150 mM NaCl, 50 mM NaF, 10 mM HEPES, pH 7.4, 2 mM EDTA, 10% (v/v) glycerol, and 5% (w/v) 2-mercaptoethanol and heated in boiling water for 3 min. The reaction was stopped by adding 0.1% SDS/1 mM CaCl_2, pH 7.4, plus 32PO_4 (0.5 μCi/ml per 60 mm dish; 1 Ci = 37 GBq) for 90 min at 37° to allow labeling of intracellular ATP (23). Prewarmed HEPES-buffered saline was added with or without the stimulating agent (4 min at 37°). The reaction was stopped by adding 1% SDS/1 mM EDTA, pH 8. Aliquots were added to concentrated SDS-PAGE buffer containing 2% SDS (w/v), 62.5 mM Tris-HCl, pH 6.8, 10% (w/v) glycerol, and 5% (w/v) 2-mercaptoethanol and heated in boiling water for 5 min. Proteins were then subjected to PAGE (24). The gels were stained with Coomassie Blue, destained, and dried, and 32P was determined by PhosphorImager analysis.

Western blot analysis. Membrane proteins were separated by PAGE and stained with Zn2+ imidazole. The gels were destained with 2% citric acid and electroblotted onto polyvinylidene difluoride membranes. After blocking, membranes typically were incubated for 2 hr with a 1:500 dilution of anti-tyrosine hydroxylase (rabbit polyclonal) followed by 1 hr of incubation with secondary antibody using a 1:1000 dilution of goat anti-rabbit IgG coupled to alkaline phosphatase. Antibody complexes were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate staining. Gel and immunoblot images for all figures were digitized using a Molecular Dynamics personal densitometer (Sunnyvale, CA).

Immunoprecipitation. Cells were stimulated as described for phosphorylation and the reaction stopped by adding stop solution containing 50 mM NaF, 1 mM EDTA, 0.1% Triton X-100, and 1 mM ATP (to prevent postlysis labeling) and freezing the samples in a dry ice/ethanol bath. After centrifugation at 13,000 x g for 5 min, supernatants were adjusted to 150 mM NaCl, 50 mM NaF, 10 mM HEPES, pH 7.4, 2 mM EDTA, 2 mM EGTA, and 2.5% Nonidet P-40 and then precleared with BRL immunoprecipitin. After incubation with BRL immunoprecipitin linked to antibody (2 hr, 25°) and washing three times with 150 mM NaCl, 50 mM NaF, 10 mM HEPES, pH 7.4, 2 mM EDTA, 0.5% Nonidet P-40, and 0.05% NaN_3, the precipitated antigen-antibody complexes were suspended in PAGE buffer, heated in boiling water for 5 min, and subjected to PAGE. 32P was digitized using a Molecular Dynamics PhosphorImager analysis.

Data analysis. Data in Table 1 and Fig. 1 were analyzed by one-way ANOVA followed by the Tukey-Kramer multiple comparison tests using GraphPad Instat (GraphPad Software, San Diego, CA). Curves were fit by nonlinear regression using GraphPad Prism.

Results

NPY inhibits EPI and NE biosynthesis but not secretion. Secretion of NE and EPI (9.9 and 13.0%, respectively, of the cell content remaining after stimulated secretion) into bovine chromaffin cell culture medium occurs after the administration of ACh (3 μM) without a concomitant decrease in the cell content of either catecholamine (Table 1). Thus, the total amounts (medium plus cellular content) of NE and EPI were greater after ACh stimulation than before stimulation (control). The stimulation of secretion could be antagonized by the prior addition of the nicotinic receptor antagonist, hexamethonium. The addition of low concentrations of NPY completely prevented the accompanying increase in catecholamine biosynthesis (the effect of 0.1 nM NPY on NE biosynthesis did not quite reach significance) but had no effect on secretion of either catecholamine (p < 0.02 and 0.001 for NE and EPI biosynthesis, respectively, by one-way ANOVA). Neither hexamethonium nor NPY alone had any effect on synthesis or secretion (not shown).

Phosphorylation of tyrosine hydroxylase. Cells were washed three times and preincubated in phosphate-free DMEM for 1 hr to deplete the endogenous phosphate. Then cells were washed three times and prelabeled in HEPES-buffered saline containing 150 mM NaCl, 10 mM HEPES, 5.5 mM d-glucose, 5 mM KCl, 1 mM MgSO_4, and 1 mM CaCl_2, pH 7.4, plus 32PO_4 (0.5 μCi/ml per 60 mm dish; 1 Ci = 37 GBq) for 90 min at 37° to allow labeling of intracellular ATP (23). Prewarmed HEPES-buffered saline was added with or without the stimulating agent (4 min at 37°). The reaction was stopped by adding 1% SDS/1 mM EDTA, pH 8. Aliquots were added to concentrated SDS-PAGE buffer containing 2% SDS (w/v), 62.5 mM Tris-HCl, pH 6.8, 10% (w/v) glycerol, and 5% (w/v) 2-mercaptoethanol and heated in boiling water for 5 min. Proteins were then subjected to PAGE (24). The gels were stained with Coomassie Blue, destained, and dried, and 32P was determined by PhosphorImager analysis.
NPY inhibits nicotine stimulation of tyrosine hydroxylase activity. Increasing concentrations of nicotine (1–100 μM) produced a concentration-dependent increase (p < 0.0001 by one-way ANOVA) in chromaffin cell tyrosine hydroxylase activity, EC_{50} = 1.2 μM (Fig. 1). Tyrosine hydroxylase activity decreased significantly when NPY (0.3 or 3 nm) was present at varying nicotine concentrations. The concentration-dependent effect of NPY was confirmed by examining the action of increasing NPY concentrations on cells stimulated with nicotine (30 μM), IC_{50} = 0.2 nm (Fig. 2). Moreover, incubation of the cells with PTX completely prevented the inhibitory effect of NPY.

NPY inhibits nicotinic-stimulated protein phosphorylation. Because tyrosine hydroxylase activity increases in response to enzyme phosphorylation (17), we hypothesized that NPY inhibits tyrosine hydroxylase activity by inhibiting phosphorylation. We first examined whether ^{32}P-labeling of chromaffin cell proteins was increased in the presence of 100 μM nicotine as demonstrated previously by others (25). Nicotine (1–100 μM) produced a significant, concentration-dependent, increase in ^{32}P incorporation into several proteins including a 60-kDa protein compared with that seen in the absence of nicotine (not shown). The addition of NPY to the culture medium prevented the nicotine-stimulated increase in ^{32}P incorporation into the 60-kDa protein (Fig. 4). The NPY effect was concentration-dependent, with half-maximal inhibition occurring at 6.4 nm.

**TABLE 1**

Effect of NPY on nicotinic receptor-stimulated catecholamine secretion and synthesis

Chromaffin cells were stimulated with ACh (3 μM) for 12 min at 37°C and secretion or cellular content of NE and EPI was measured by electrochemical detection after liquid chromatography. Hexamethonium (C6) (300 μM), NPY (0.1 nm) or (10 nm) were added with ACh. Neither hexamethonium nor NPY had any effect on secretion occurring in the absence of ACh. Data are presented as the mean ± standard error of triplicate determinations and are representative of three individual experiments with similar results. Significance was determined by the Tukey-Kramer multiple comparisons test.

<table>
<thead>
<tr>
<th>Condition</th>
<th>NE</th>
<th>EPI</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Cell</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.6 ± 0.3</td>
<td>14.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>ACh</td>
<td>2.2 ± 0.2^a</td>
<td>16.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>ACh + C6</td>
<td>0.8 ± 0.3^b</td>
<td>14.9 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>ACh + NPY1</td>
<td>2.1 ± 0.1^a</td>
<td>14.4 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>ACh + NPY2</td>
<td>2.0 ± 0.2^a</td>
<td>13.4 ± 0.1^d</td>
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Note: All values are in nmol/10^6 cells. *Significantly different from control (p < 0.001).
^a Significantly different from control (p < 0.01).
^b Significantly different from ACh alone (p < 0.001).
^c Significantly different from ACh alone (p < 0.01).
^d Significantly different from ACh alone (p < 0.05).
NPY decreases 32P content of immunoprecipitated tyrosine hydroxylase. Western blot analysis revealed that tyrosine hydroxylase co-migrates with the phosphorylated 60-kDa protein, suggesting that the 60-kDa protein is tyrosine hydroxylase (not shown). We confirmed the identity of the 60-kDa band as tyrosine hydroxylase by immunoprecipitation. The concentration of the 60-kDa protein was not altered by the addition of nicotine or nicotine and NPY (Fig. 5A), whereas the nicotine-induced increase in 32P content decreased with increasing NPY concentrations (Fig. 5B). The concentration of NPY for half-maximal inhibition of phosphorylation determined by immunoprecipitation was 3.2 ± 1.4 nM (not shown).

**NPY and related peptides inhibit tyrosine hydroxylase phosphorylation through a PTX-sensitive process.** Incubation of chromaffin cells with PTX completely prevented the NPY inhibition of the 60-kDa protein phosphorylation (Fig. 4). A decrease in 32P content of the 60-kDa protein attributable to NPY-induced changes in protein content of the gels was ruled out by Western blot analysis (Fig. 6). Examination of the inhibitory effect of NPY and related peptides on nicotine stimulation of 60-kDa protein phosphorylation provided a rank order of potency of [Leu31,Pro34]NPY ≥ NPY ≥ NPY18–36 (Fig. 7).
The addition of low concentrations of NPY to the chromaffin cells results in increased catecholamine biosynthesis in a concentration-dependent fashion. The amount of catecholamine secreted plus synthesized after nicotinic receptor stimulation, in the presence of NPY (10 nM), is not significantly different from the total in the absence of cholinergic receptor stimulation. Correspondingly, the cellular content of either catecholamine is significantly less after nicotine plus NPY compared with nicotine alone. Thus the presence of low concentrations of NPY in the culture media selectively antagonizes the increase in catecholamine biosynthesis but not the increase in secretion.

The lack of an NPY effect on catecholamine secretion is in conflict with data presented by others (15, 26). We investigated this issue in detail and demonstrated that high NPY concentrations can inhibit chromaffin cell [3H]NE secretion (13, 16). However, they act by blocking the nicotinic receptor-gated ion channel rather than via one of the known NPY receptor subtypes (27). The effects of NPY on secretion most likely represent a pharmacological rather than a physiological phenomenon. Our studies are supported by the recent findings that NPY does not inhibit bovine chromaffin cell IC$_{Ca}$ as measured by the patch-clamp technique (28).

Nicotinic receptor stimulation increases neuronal catecholamine biosynthesis through an effect on the rate-limiting enzyme, tyrosine hydroxylase (17). Increasing concentrations of nicotine, that are maximal at 1 x 10$^{-5}$ M, produce a corresponding increase in chromaffin cell tyrosine hydroxylase activity. The inclusion of NPY with increasing nicotine concentrations inhibits tyrosine hydroxylase activity via a PTX-sensitive process, IC$_{50}$ = 0.2 nM. NPY decreases the efficacy of nicotine stimulation, suggesting a non- or uncompetitive action. The potency of NPY as a tyrosine hydroxylase inhibitor is in good agreement with the ability of NPY to displace 125I-NPY from chromaffin cell membranes, IC$_{50}$ = 0.9 nM, (GTP-sensitive) as well as to inhibit forskolin-stimulated cAMP accumulation, IC$_{50}$ = 0.27 nM (PTX-sensitive) (14).

NPY fragments and related peptides produce a rank order of potency that is characteristic of the Y1 receptor subtype, i.e., NPY $\geq$ [Leu$^{25}$,Pro$^{34}$]NPY $\geq$ [Leu$^{25}$,Pro$^{34}$]NPY $\geq$ NPY$_{18-36}$. These data agree with our previous characterization of the chromaffin cell NPY receptor as a Y1 subtype (14). Another report has referred to the chromaffin cell NPY receptor as a Y3 subtype (29). Our data show that peptide YY is nearly as effective an inhibitor of tyrosine hydroxylase activity and 125I-labeled NPY binding as NPY (14), observations that distinguish it clearly from a Y3 receptor subtype (8).

Because tyrosine hydroxylase activity can be increased by phosphorylation through various mechanisms (17), we next elected to determine whether the decrease in tyrosine hydroxylase activity could be attributed to decreased enzyme phosphorylation. Increasing NPY concentrations produce a concentration-dependent decrease in $^{32}$P incorporation into a 60-kDa protein that co-migrates with tyrosine hydroxylase, IC$_{50}$ = 6.4 nM, suggesting that the 60-kDa protein is tyrosine hydroxylase. Immunoprecipitation with anti-tyrosine hydroxylase coupled to BRL immunoprecipitin, confirmed the identification of the 60-kDa protein as tyrosine hydroxylase. The effect of NPY on enzyme phosphorylation is PTX-sensitive. NPY fragments and related peptides produce a rank order of potency that is also characteristic of the Y1 receptor subtype, i.e., [Leu$^{13}$,Pro$^{34}$]NPY $\gg$ NPY $\gg$ NPY$_{18-36}$. The right shift in the dose-response curve for inhibition of phos-
phorylation, compared with inhibition of tyrosine hydroxylase activity may be related to enzyme phosphorylation by protein kinases not inhibited by NPY.

These data demonstrate that NPY inhibits cholinergic receptor stimulation of tyrosine hydroxylase activity by decreasing enzyme phosphorylation. However, the mechanism by which NPY inhibits tyrosine hydroxylase phosphorylation has not been described and is subject to speculation. Because NPY can inhibit Ca$^{2+}$ currents in sensory (30) and myenteric neurons (31) inhibition of phosphorylation at Ser$^{19}$ by Ca$^{2+}$/calmodulin-dependent protein kinase II (32–34) could be the mechanism through which NPY can act in chromaffin cells. However, if NPY can inhibit this enzyme it would not occur through an effect on Ca$^{2+}$ influx through voltage-operated calcium channels because the peptide does not block $^{45}$Ca$^{2+}$ into chromaffin cells through these channels (16). Moreover, the nicotinic receptor-gated ion channel is probably not the site of NPY action because much higher concentrations (1000-fold) of NPY are required to inhibit $^{45}$Ca$^{2+}$ influx through the ion channel than to inhibit tyrosine hydroxylase activity (16).

An alternative site for NPY action could be the Ca$^{2+}$-activated adenylate cyclase, which has been shown to be active in chromaffin cells (35). In this model, nicotinic receptor stimulation results in increased intracellular Ca$^{2+}$, enzyme activation, and stimulation of protein kinase A activity. By activating Gs, NPY would inhibit activity of this enzyme through an effect on adenylate cyclase similar to the effect of NPY on forskolin-stimulated cAMP accumulation (14). This possibility seems unlikely, as well, because attempts to demonstrate NPY inhibition of nicotinic as well as noncholinergic receptor-stimulated cAMP accumulation have been unsuccessful.\(^1\)

Other possible mechanisms include the activation of an inward rectifier K$^+$ channel or a protein phosphatase. NPY, acting through Y1 receptors, can activate a cloned G protein-coupled inward rectifier K$^+$ channel expressed in Xenopus laevis oocytes (36). Activation of this K$^+$ current would stabilize the membrane and reduce Ca$^{2+}$ influx. Guinea pig chromaffin cells contain such a channel (37). Alternatively, NPY could activate protein phosphatase 2A, which has been implicated in the dephosphorylation of tyrosine hydroxylase (38). No data are available to rule out either of these two possibilities. Thus, further studies are required to determine the mechanism by which NPY inhibits tyrosine hydroxylase phosphorylation.

These data raise at least two questions relative to the physiological significance of these observations. First, what is the temporal relationship between nicotinic receptor stimulation, the activation of tyrosine hydroxylase, and the secretion of NPY? The answer to this question can be inferred from what is known about neuropeptide storage and secretion. Chromaffin cell neuropeptides are stored in large dense-cored vesicles as opposed to small dense-cored vesicles (39). Classical neurotransmitters such as the biogenic amines are rapid but short acting agents that are co-stored and co-released with neuropeptides from chromaffin cells. Neuropeptides may exert a slower but more sustained action than the low molecular weight classical neurotransmitters (40). Thus NPY would act in a prolonged manner, exerting its effect on chromaffin cell tyrosine hydroxylase sometime after the initial ACh action. NPY would facilitate the return of tyrosine hydroxylase activity to basal values.

Second, is chromaffin cell NPY secreted in amounts sufficient to inhibit nicotinic receptor-stimulated cAMP accumulation and tyrosine hydroxylase activation? The answer to this question can be inferred from the observation that prolonged nicotine stimulation of chromaffin cells increases the concentration of NPY in conditioned medium to 0.6 nm (13), which is near the IC50 for inhibition of $^{125}$I-NPY binding (14), cAMP accumulation (14) and tyrosine hydroxylase activity. Studies are in progress using a specific NPY antibody, as well as specific NPY receptor antagonists, to demonstrate that NPY present in chromaffin cell-conditioned media after nicotinic receptor stimulation inhibits tyrosine hydroxylase activation.

NPY is a potent and abundant neuropeptide that satisfies the criteria for classification as a neurotransmitter (1). Its distribution throughout the nervous system suggests a fundamental role in neurotransmission. Indeed, activity as a vasoconstrictor, anxiolytic agent, and inducer of food intake supports this contention. Although NPY has been shown to alter second messenger production, no information has been provided as to the consequences of these alterations. The data presented here demonstrate that NPY can inhibit catecholamine biosynthesis through a receptor-mediated process, which results in the inhibition of tyrosine hydroxylase phosphorylation and a corresponding decrease in enzyme activity.

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References


\(^1\) J. Zheng and T. D. Hexum, unpublished observations.

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