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Stimulation of Tyrosine Hydroxylase Gene Transcription Rate by Nicotine in Rat Adrenal Medulla

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SUMMARY

The administration of nicotine stimulates the transcription rate of the tyrosine hydroxylase gene in rat adrenal medulla. This stimulation occurs very rapidly (within 10 min) after the subcutaneous injection of nicotine and persists for at least 1 hr after a single injection of the drug. Repeated injections of the drug (seven injections once every 30 min) are associated with a more persistent activation of the gene (for at least 3 hr) and elicit the induction of tyrosine hydroxylase mRNA and tyrosine hydroxylase protein. Quantitatively, the increases in tyrosine hydroxylase gene transcription rate, mRNA, and protein are approximately equivalent. The effect of nicotine is dose dependent; a significant increase in tyrosine hydroxylase gene transcription rate is observed using 1.0 mg/kg nicotine, whereas 0.33 mg/kg nicotine produces no effect. The nicotinic receptor antagonists hexamethonium and mecamylamine partially inhibit the nicotine-mediated stimulation of the tyrosine hydroxylase gene. The lack of total blockade of the nicotine-mediated effect suggests that nicotine acting centrally may elicit the release of substances from the splanchnic nerve, that interact with receptors (other than the nicotinic receptor) that play a role in regulating the tyrosine hydroxylase gene. The administration of carbachol also stimulates rat adrenomedullary tyrosine hydroxylase gene transcription rate. The effect of carbachol is not inhibited by hexamethonium but is completely blocked by the muscarinic antagonist atropine. The muscarinic agonist bethanechol also stimulates this gene in rat adrenal medulla. Our results suggest that multiple receptors and signal transduction pathways are involved in the regulation of the tyrosine hydroxylase gene in the rat adrenal medulla.

Tyrosine hydroxylase [tyrosine 3-monooxygenase; L-tyrosine:tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2] in the rat adrenal medulla is induced *in vivo* by prolonged stress and other physiological stimuli that activate the sympathetic nervous system (1–3). In addition, treatment of animals with catecholamine-depleting drugs like reserpine results in the induction of adrenal tyrosine hydroxylase (4); these drugs reflexively increase sympathetic tone. Adrenal TH-mRNA is also elevated in rats treated with reserpine or exposed to prolonged stress (5–7). This elevation of adrenal TH-mRNA precedes and presumably is responsible for the induction of tyrosine hydroxylase enzyme. It is not yet known whether the elevation of TH-mRNA is due to an increase in the transcription rate of the tyrosine hydroxylase gene or a decrease in the degradation rate of TH-mRNA in the adrenal medulla.

The induction of tyrosine hydroxylase by these stimuli is blocked by transection of the splanchnic nerve, which innervates the adrenal medulla (8, 9). Hence, the induction of this adrenal enzyme is thought to be mediated by neurotransmitters that are released from splanchnic nerve terminals. However, the identities of these neurotransmitters have not been clearly established. Recently, Stachowiak *et al.* (10) presented evidence that the nicotinic cholinergic receptor blocker chlorisondamine inhibits the induction of adrenal TH-mRNA elicited by cold stress. This evidence suggests that acetylcholine released from the splanchnic nerve stimulates nicotinic receptors on adrenal chromaffin cells, resulting in subsequent induction of tyrosine hydroxylase. However, earlier results from two different laboratories (11, 12) contradict this hypothesis. These workers found that nicotinic receptor antagonists block the reserpine-mediated induction of tyrosine hydroxylase in the superior cervical ganglion but not in the adrenal medulla. Because these previous studies examined the effects of nicotinic receptor antagonists on adrenal medullary events that occur many hours or days after the application of the drugs, it is difficult to interpret the results unequivocally. Hence, the involvement of nicotinic receptors in the regulation of adrenal tyrosine hydroxylase gene expression requires further study.

In the present study, we have investigated 1) whether tyro-

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ABBREVIATIONS: TH-mRNA, tyrosine hydroxylase mRNA; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair(s); SDS, sodium dodecyl sulfate.

sine hydroxylase gene transcription is elevated in rats treated with nicotine, 2) whether this elevation leads to the induction of TH-mRNA and tyrosine hydroxylase enzyme, and 3) whether nicotinic receptors are involved. Our results demonstrate that a single injection of nicotine rapidly but transiently activates the tyrosine hydroxylase gene. However, TH-mRNA and tyrosine hydroxylase enzyme levels are induced only when the transcription rate of the gene is elevated for at least 3 hr by repeated injections of nicotine. Furthermore, the activation of the tyrosine hydroxylase gene is only partially inhibited by nicotinic receptor antagonists. Our results suggest that a number of mechanisms may be involved in regulating tyrosine hydroxylase gene transcription in the rat adrenal medulla.

Experimental Procedures

Materials. [α-^{32}P]UTP and ^{125}I-Protein A were purchased from ICN Biomedicals (Costa Mesa, CA). [α-^{32}P]dCTP was purchased from Amersham Corporation (Arlington Heights, IL). [1-^{14}C]Tyrosine was purchased from New England Nuclear Corporation (Boston, MA). All drugs were purchased from Sigma Chemical Co. (St. Louis, MO), except for mecamylamine, which was a kind gift from Dr. Leo Abood (Department of Pharmacology, University of Rochester Medical Center, Rochester, NY). All other chemicals were of the highest purity available from commercial sources.

pTH.4 is a recombinant plasmid containing approximately 400 bp encoding the 3′ end of rat TH-mRNA, and it was isolated as described by Lewis *et al.* (13). The *Pst*I-*Kpn*I restriction fragment of pTH.4 contains 280 bp, corresponding to bp 1240 to bp 1521, of the rat TH cDNA sequence (14). pTHg6.3 is a recombinant plasmid containing approximately 6300 bp encoding the tyrosine hydroxylase gene. The genomic DNA was isolated in our laboratory from a rat liver genomic library constructed in Charon 35. It encodes sequences of the tyrosine hydroxylase gene extending from approximately −800 bp upstream from the transcriptional start site (at the *Kpn*I site) and extending downstream to the *Eco*RI site in exon 11 of the coding region of the gene. This cloned segment of the tyrosine hydroxylase gene was inserted into pGEM7Zf and designated pTHg6.3. Extensive restriction mapping and Southern analysis suggest that this rat genomic clone is similar, if not identical, to tyrosine hydroxylase genomic clones reported by other workers (15, 16). The restriction sites noted above correspond to those reported by Brown *et al.* (16). pACT1.1 is a recombinant plasmid containing 1140 bp of the coding region of the mouse α-actin cDNA [the *Pst*I restriction fragment described by Minty *et al.* (17)] cloned into pSP64. This probe was a kind gift from Dr. Elena Ciejek (University of Rochester, Rochester, NY).

Treatment of animals. Male Sprague-Dawley rats (150–200 g) were purchased from Charles River Company and permitted free access to food and water for at least 24 hr before treatment. Drugs were administered as follows: nicotine (doses are presented as nicotine base, even though the drug was administered as the bitartrate salt), 0.33–3.3 mg/kg subcutaneously; hexamethonium, 15 mg/kg intraperitoneally; mecamylamine, 15 mg/kg intraperitoneally; carbachol, 1 mg/kg intraperitoneally; bethanechol, 10 mg/kg intraperitoneally; atropine, 30 mg/kg intraperitoneally; reserpine, 10 mg/kg subcutaneously; and picrotoxin, 2 mg/kg intraperitoneally. All drugs were dissolved in phosphate-buffered saline (10 mM potassium phosphate, pH 7.5, 150 mM NaCl) and the pH was adjusted to 7.4, except for reserpine, which was dissolved in 20% ascorbic acid. Control animals were injected with an identical volume (1 ml/kg) of vehicle. At the appropriate time after drug treatment, the animals were rapidly anesthetized using 150 mg/kg pentobarbital (intraperitoneally), and adrenal glands were removed under anesthesia 3–4 min later. Pentobarbital was used to anesthetize and kill the animals because it was shown to produce minimal stimulation, by itself, of the adrenal gland (18, 19).

Tyrosine hydroxylase assay. Tyrosine hydroxylase activity was assayed as described by Tank *et al.* (18). Adrenal glands were removed, rapidly frozen on dry ice, and stored at −70°. All subsequent procedures were performed at 4°. Frozen adrenal glands were homogenized in 250 μl of 30 mM potassium phosphate (pH 6.8), 50 mM NaF, 10 mM EDTA, and the homogenate was centrifuged at 20,000 × *g* for 15 min. In order to remove endogenous catecholamines and other small molecules that might interfere with the assay, the supernatant was subjected to gel filtration, using Sephadex G-50 columns equilibrated with 30 mM potassium phosphate (pH 6.8), 10 mM NaF, 0.1 mM EDTA. A 50-μl aliquot of the gel-filtered supernatant was used for the assay. Reaction mixtures contained (final concentrations) 100 mM potassium phosphate (pH 6.8), 0.2 mM 6-methyl-5,6,7,8-tetrahydropterine, 5 mM sodium ascorbate, 1600 units of catalase, 10 mM EDTA, and 0.1 mM [1-^{14}C]tyrosine, in a final volume of 100 μl. Reactions were carried out for 10 min at 30°. The reactions were terminated by chilling in an ice-water bath, and the resulting [1-^{14}C]dihydroxyphenylalanine was decarboxylated by the addition of 20 μl of 1 M sodium ferricyanide (20). After incubation at 37° for 30 min, the $^{14}CO_2$ was collected in wells containing 0.2 ml of TS-1 tissue solubilizer. The wells were suspended from rubber stoppers that capped the reaction tubes. The radiolabeled CO_2 was collected for 60 min at 37°, and the radioactivity in the wells was measured by liquid scintillation counting. Protein was measured by the method of Bradford (21). Results were expressed as nmol of $^{14}CO_2$ formed/min/mg of protein.

Measurement of TH-mRNA. Total cellular RNA from adrenal glands was isolated by the acid-guanidinium-phenol-chloroform method of Chomczynski and Sacchi (22). RNA concentration was estimated using absorbance at 260 nm (1 absorbance unit = 40 μg/ml). TH-mRNA was measured by hybridization to the radiolabeled 280-bp *Pst*I-*Kpn*I restriction fragment of pTH.4 (13). In order to detect degradation of TH-mRNA in the adrenal RNA samples, Northern blot analysis was performed as previously described (5, 23). TH-mRNA was routinely quantitated by dot hybridization analysis, as previously described (5, 23). To normalize the TH-mRNA signals between experiments in the dot hybridizations, adrenal TH-mRNA values were compared with values obtained from a standard curve using known amounts of TH-cRNA sense strand synthesized from a Gemini plasmid containing the sequences of TH-mRNA present in the cDNA probe. The TH-mRNA level in a sample was expressed as pg of TH-cRNA sense strand equivalents/μg of total cellular RNA spotted onto the nitrocellulose. The cDNA was radiolabeled with [^{32}P]phosphate by the random priming procedure of Feinberg and Vogelstein (24).

Nuclear run-on assays. After removal of the adrenal glands from anesthetized rats, adrenal medullae were carefully dissected free from the adrenal cortices, with the aid of a dissecting microscope. Whole adrenal medullae were isolated by this dissection; however, no attempt was made to recover quantitatively all of the adrenal cortical tissue when it was used for the run-on assays. Nuclei were then isolated from the medullae and, in a few experiments, from the cortices (see Table 1), as described by McKnight and Palmiter (25). Nuclei isolated from two adrenal medullae or two adrenal cortices were suspended in 100 μl of 50% glycerol, 0.1 mM EDTA, 5 mM $MgCl_2$, 50 mM HEPES (pH 7.5), and mixed with 100 μl of a reaction mixture containing 0.2 M KCl, 8 mM dithiothreitol, 1 mM GTP, 1 mM CTP, 1 mM ATP, [α-^{32}P]UTP (600 Ci/mmol, 0.2 mCi/assay), 0.8 mg/ml creatine kinase, 17.6 mM creatine phosphate, and 100 mM HEPES (pH 7.5). The assay was carried out at 24° for 30 min and terminated by chilling in an ice-water bath. The radiolabeled RNA was isolated as described by McKnight and Palmiter (25). An aliquot of the RNA was removed for measurement of incorporation of radiolabeled UTP into total RNA (column 1 in Table 1). This measurement was made using DE81 filter paper, as described by Sambrook *et al.* (26). The remaining RNA was subjected to gel filtration using Sephadex G-50 spun columns to remove unincorporated [α-^{32}P]UTP.

The amount of radiolabeled TH-RNA synthesized in the run-on assay was measured by hybridization to a cloned tyrosine hydroxylase genomic probe, pTHg6.3, bound to a nitrocellulose filter. pGEM7Zf

bound to nitrocellulose filters was used as a control for hybridization of radiolabeled RNA to bacterial sequences. In some experiments, radiolabeled RNA was hybridized to nitrocellulose filters containing pACT1.1. This probe was used to measure the rate of actin gene transcription. The hybridizations were carried out using 2.4 μg of plasmid DNA bound to the nitrocellulose filters. Greater amounts of plasmid DNA bound to the filters did not produce an increase in signal. Furthermore, varying the input cpm of RNA added to the hybridization mixtures from 0.26 to 1.1 × 10^6 cpm did not significantly affect the calculated values for the relative transcription rate of the TH gene. Hybridization efficiencies were routinely performed by including a known amount of [^{3}H]cRNA sense strand (synthesized from the pTHg6.3 plasmid) in the hybridization reactions. Hybridization efficiencies ranged from 60 to 80% in different experiments; however, the variability in hybridization efficiency between hybridization reactions within each experiment was <5%. The relative transcription rates reported in Results were not corrected for these small differences in hybridization efficiency.

Hybridizations were carried out under the conditions described by McKnight and Palmiter (25). Filters were hybridized to radiolabeled RNA for 3 days at 45° and then washed once for 2 hr and once for 30 min at 45° with buffer containing 0.3 M NaCl, 3 mM EDTA, 10 mM Tris (pH 7.5), and 0.1% SDS. The filters were then either dried or subjected to RNase treatment (10 μg/ml RNase A for 30 min at 37°) to remove radiolabeled sequences that were not perfectly complementary to plasmid DNA sequences. Radioactivity that remained hybridized to each filter was measured by Cerenkov counting. Filters were treated as described by McKnight and Palmiter (25), to elute radiolabeled cRNA sense strand for determination of hybridization efficiency.

The radiolabeled RNA that hybridized to each filter was divided by the total amount of radiolabeled RNA added to the hybridization reactions (input cpm) and was expressed as ppm. The relative transcription rate of the gene for tyrosine hydroxylase was calculated as the difference in the radioactivity hybridized to filters with and without TH-cDNA insert.

Western blot analysis of tyrosine hydroxylase protein. Adrenal supernatants were isolated and subjected to Sephadex G-50 gel filtration as described above. Adrenal supernatant proteins (25–100 μg) were separated on 10% SDS-polyacrylamide gels (18) and then transferred to nitrocellulose using a Bio-Rad Transblot apparatus, as described by Towbin *et al.* (27). After transfer, the nitrocellulose sheets were incubated for 1 hr with BLOTTO buffer (5% nonfat dry milk, 0.05% Triton X-100, 2 mM $CaCl_2$, 0.02% NaN_3, 50 mM Tris·HCl, pH 7.4) to saturate nonspecific binding sites and then incubated for 2 hr with antiserum to tyrosine hydroxylase (1/3000 dilution of whole serum), dissolved in BLOTTO buffer. The antiserum was produced, in rabbits, against tyrosine hydroxylase purified from rat pheochromocytoma tissue (18) and was shown in numerous studies to be specific for tyrosine hydroxylase (18, 28, 29). The nitrocellulose sheets were then washed three times for 10 min with 0.05% Triton X-100, Tris-saline buffer (10 mM Tris, pH 7.5, 150 mM NaCl), and then incubated for 30 min with ^{125}I-Protein A dissolved in BLOTTO buffer. Finally, the blots were washed once for 3 min in the 0.05% Triton X-100/Tris-saline buffer and two times for 10 min in Tris-saline buffer. The blots were exposed to X-ray film for 1–3 days, and the density of the tyrosine hydroxylase bands was measured by densitometry.

A known amount of purified tyrosine hydroxylase was electrophoresed in a separate lane of each gel. The density reading for tyrosine hydroxylase protein in each adrenal sample was divided by the density reading obtained for purified tyrosine hydroxylase on the same autoradiogram, to normalize between different blots and experiments. As a control to check for the specificity of the antiserum, an excess of purified tyrosine hydroxylase was incubated with the antiserum overnight at 4°. The resulting immunoprecipitate was removed by centrifugation (18), and the supernatant antiserum, which no longer possessed tyrosine hydroxylase antibodies, was used for the Western analysis. A tyrosine hydroxylase band was not obtained in the rat adrenal supernatants analyzed by Western analysis using this preabsorbed antiserum (see Fig. 5, *lane 10*). The density readings for tyrosine hydroxylase protein in each adrenal supernatant, normalized as described above, were plotted against the μg of total protein added to the appropriate lanes of the gel, and the tyrosine hydroxylase protein values were expressed as the slope of the linear portion of this line (i.e., normalized density units/μg of total protein). The density readings representing tyrosine hydroxylase protein were linear up to 50–75 μg of protein for adrenal supernatants isolated from control animals, whereas these tyrosine hydroxylase protein values were linear up to only 25–50 μg of total protein for adrenal supernatants isolated from nicotine-treated animals (Fig. 5B).

Statistical analyses. Unless otherwise noted, the data were statistically analyzed using one-way and two-way analysis of variance, where appropriate. Comparisons between different groups were made using either the Student-Neumann-Kuels test or the least significant difference test (30). A level of $p < 0.05$ was considered statistically significant.

Results

Effect of nicotine on the transcription rates of the tyrosine hydroxylase and actin genes in rat adrenal medulla and adrenal cortex, measured by a nuclear run-on assay. The transcription rates of the tyrosine hydroxylase and actin genes in nuclei isolated from rat adrenal medulla and adrenal cortex were estimated using a nuclear run-on assay. Because reinitiation of RNA polymerase II-dependent transcription is very low in isolated nuclei (31), this assay is thought to measure primarily the "run-on" synthesis of RNA molecules catalyzed by RNA polymerases that formed initiation complexes with specific genes in the intact tissue. Hence, the nuclear run-on assays provide an estimate of the number of RNA polymerases bound to a specific gene (which is directly proportional to the rate of transcription of the gene) in the intact tissue. Preliminary studies demonstrated that using 0.2 mCi of [α-^{32}P]UTP in the assay provided a large enough signal such that a pair of adrenal medullae from a single animal could be used to measure tyrosine hydroxylase and actin gene transcription rates accurately.

The pmol of UTP incorporated into nascent RNA chains using nuclei isolated from adrenal medullae was 40–50% of that incorporated into RNA using nuclei isolated from adrenal cortices (Table 1A). Due to the low yield of nuclei isolated from two adrenal medullae and to the technical difficulty of accurately obtaining a small volume of isolated nuclei for measurement of DNA, the pmol of UTP incorporated into RNA/mg of DNA was not routinely measured. However, in select experiments this parameter was determined and found to be almost identical for nuclei isolated from either adrenal medulla or adrenal cortex (15 ± 2 pmol of UTP/mg of DNA, six rats for adrenal cortical nuclei; 19 ± 2 pmol of UTP/mg of DNA, 13 rats for adrenal medullary nuclei). Hence, the greater incorporation of radiolabeled UTP into RNA using cortical nuclei, relative to that observed using medullary nuclei, was presumably due to the larger amount of tissue obtained from adrenal cortices, compared with adrenal medullae. Nicotine treatment did not significantly affect the incorporation of radiolabeled UTP into total RNA in either the medullary or cortical nuclei.

As observed in Table 1A, hybridization of radiolabeled nuclear RNA transcripts to pGEM7Zf was relatively low. Similar hybridization values were obtained when pBR322, pSP64, or pGEM3Zf DNA was utilized to measure background hybridization to bacterial plasmid sequences. Hence, pGEM7Zf was

TABLE 1

Effect of nicotine on the rates of transcription of the tyrosine hydroxylase and actin genes in rat adrenal medulla and cortex

Rats were injected subcutaneously with either saline or 3.3 mg/kg nicotine, and adrenal glands were removed under anesthesia 20 min after the injection. Adrenal medullae and cortices were dissected free from one another, and nuclei were isolated from both tissues. Nuclear run-on assays and hybridizations were performed as described in the text, using pTHg6.3 and pACT1.1 to measure radiolabeled RNA transcripts coding for tyrosine hydroxylase and actin, respectively. pGEM7Zf was used to measure nonspecific hybridization to bacterial sequences. The amount of radiolabeled UTP incorporated into total nascent RNA during the run-on assay was expressed as the pmol incorporated/pair of either adrenal medullae or adrenal cortices (quantitative recovery of adrenal cortical tissue was not attempted). The cpm hybridized to different filter-bound plasmid DNAs were divided by the cpm of radiolabeled RNA added to the hybridizations (input cpm) and expressed as ppm. Relative transcription rates were calculated as the ppm hybridized to either pTHg6.3 or pACT1.1 DNA minus the ppm hybridized to pGEM7Zf. A, Filters were washed and radioactivity was measured without RNase treatment. B, Filters were washed and treated with RNase before measurement of radioactivity. The results represent the means ± standard errors from eight to 13 animals for the tyrosine hydroxylase gene transcription data and from four to six animals for the actin gene transcription data.

A. Before RNase treatment	UTP incorporated	Input	Hybridization to			Relative transcription rate	
			pTHg6.3	pACT1.1	pGEM7Zf	TH	Actin
	pmol	*cpm* (×10⁶)	*ppm*			*ppm*	
Adrenal medulla							
Saline	0.51 ± 0.04	0.27 ± 0.02	455 ± 35	271 ± 30	93 ± 10	363 ± 31	172 ± 25
Nicotine	0.46 ± 0.04	0.41 ± 0.05	833 ± 60[a]	333 ± 16	162 ± 22	721 ± 44[a]	146 ± 8
Adrenal cortex							
Saline	1.3 ± 0.07	1.0 ± 0.15	165 ± 25	243 ± 49	82 ± 22	83 ± 18	114 ± 44
Nicotine	1.1 ± 0.15	0.79 ± 0.12	196 ± 25	274 ± 42	81 ± 16	114 ± 13	161 ± 37

B. After RNase treatment	Hybridization to		Relative transcription rate
	pTHg6.3	pGEM7Zf	
	ppm		*ppm*
Adrenal medulla			
Saline	278 ± 31	42 ± 5	235 ± 27
Nicotine	622 ± 51[a]	96 ± 15	527 ± 40[a]
Adrenal cortex			
Saline	50 ± 10	38 ± 11	11 ± 2
Nicotine	87 ± 21	48 ± 12	40 ± 11[a]

[a] $p < 0.01$, compared with saline-treated controls.

used routinely in these experiments. The radioactivity that hybridized to filters containing either pTHg6.3 or pACT1.1 was at least twice that which hybridized to filters containing pGEM7Zf. Hence, the tyrosine hydroxylase-specific and actin-specific signals obtained from these assays could be quantitated accurately and reliably.

The relative transcription rates for the actin gene were similar in nuclei isolated from either adrenal medulla or adrenal cortex (Table 1A). Nicotine did not significantly affect actin gene transcription in either tissue. As expected, tyrosine hydroxylase gene transcription rate was much greater in nuclei isolated from adrenal medulla, compared with those isolated from adrenal cortex. This difference was particularly apparent when the filters were treated with RNase to lower background hybridization and to destroy hybrids that were not totally complementary. Under these assay conditions, the relative transcription rate of the tyrosine hydroxylase gene in adrenal cortex was close to background signal (Table 1B). In contrast, RNase treatment lowered the tyrosine hydroxylase gene transcription signal by only 30–40% using nuclei isolated from adrenal medulla. Hence, most of the adrenal medullary nuclear radiolabeled RNA that hybridized to pTHg6.3 was perfectly complementary to the TH gene.

Nicotine treatment was associated with a 2–3-fold increase in the relative transcription rate of the tyrosine hydroxylase gene in the adrenal medullary nuclei (Table 1). This increase was observed whether or not the filters were treated with RNase. However, in all subsequent experiments, the signals obtained after RNase treatment of the filter-bound hybrids were used as estimates of the transcription rate of the tyrosine hydroxylase gene.

Time course of the effect of a single injection of nicotine on tyrosine hydroxylase gene transcription in rat adrenal medulla. Rats were injected with either nicotine (3.3 mg/kg) or saline, and adrenals were removed under anesthesia at different times after treatment. The relative transcription rate of the tyrosine hydroxylase gene did not change significantly over a 3-hr period in rats injected with saline (Fig. 1). In rats treated with nicotine, a 2–3-fold increase in tyrosine hydroxylase gene transcription rate was observed (Fig. 1). This increase in transcription rate occurred very rapidly, being maximal 10 min after the injection of nicotine, and persisted for at

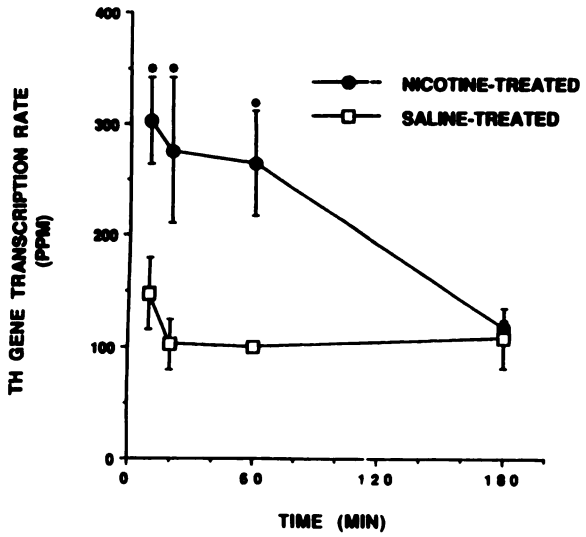

Fig. 1. Time course of the effect of a single injection of nicotine on adrenal medullary tyrosine hydroxylase gene transcription rate. Rats were injected subcutaneously with saline or 3.3 mg/kg nicotine, and adrenal glands were removed under anesthesia at the designated time points. The results are presented as the means ± standard errors from five or six animals. *, $p < 0.05$, compared with saline-treated controls.

least 1 hr after administration of the drug. However, the effect was transient, in that tyrosine hydroxylase gene transcription in nicotine-treated rats was identical to that observed in saline-treated rats 3 hr after the injection.

Effects of different concentrations of nicotine, injected either once or repeatedly, on adrenal tyrosine hydroxylase gene transcription rate. In one set of experiments, adrenal medullary tyrosine hydroxylase gene transcription rate was measured 20 min after single injections of either saline or different doses of nicotine. A maximal 2–3-fold elevation of transcription rate was observed using either 2.3 mg/kg or 3.3 mg/kg nicotine (Fig. 2A). Both of these high doses of nicotine elicited convulsions that began 1–2 min after injection and persisted for approximately 1 min, after which the animals remained sedated. The lower doses of nicotine (0.33 and 1.0 mg/kg) did not elicit convulsions. Nevertheless, treatment with 1.0 mg/kg nicotine was associated with a significant increase (approximately 2-fold) in tyrosine hydroxylase gene transcription rate. The lower dose of nicotine (0.33 mg/kg) did not produce a significant effect on transcription rate.

In a second set of experiments, rats were administered different doses of nicotine once every 30 min for 3 hr (seven injections) and adrenal medullary tyrosine hydroxylase gene transcription rate was measured 20 min after the last injection. Using this paradigm, the rats convulsed, as described above, after the first injection of either 2.3 or 3.3 mg/kg nicotine; however, subsequent injections did not elicit convulsions. Surprisingly, the basal tyrosine hydroxylase gene transcription rate was lower in animals injected repeatedly with saline than in those injected only once with saline (Fig. 2 legend). This effect might be due to the animals becoming accustomed to the handling and injections during the repeated injection paradigm. This conclusion suggests that the adrenal medullary tyrosine hydroxylase gene transcription rate is slightly elevated in animals injected only once with saline, presumably due to the initial stress of the injection. When nicotine was administered repeatedly every 30 min for 3 hr, 2–3-fold increases in adrenal medullary tyrosine hydroxylase gene transcription rate were observed using doses of nicotine of ≥1.0 mg/kg (Fig. 2A).

Effect of nicotine on adrenal TH-mRNA levels. A single injection of nicotine (2.3 or 3.3 mg/kg) did not elicit measurable increases in TH-mRNA levels at time points between 1 hr and 48 hr after injection (data not shown). However, repeated injections of nicotine once every 30 min for 3 hr were associated with dose-dependent increases in adrenal TH-mRNA levels (Figs. 2B and 3). A Northern blot of adrenal TH-mRNA is presented in Fig. 3A. It is obvious from this Northern analysis that 2.3 mg/kg nicotine elicited a significant increase in adrenal TH-mRNA levels. Dot blot hybridizations (an example is provided in Fig. 3B) were used to quantitate these increases. TH-mRNA levels were induced 2–3-fold by doses of nicotine of ≥1.0 mg/kg (Figs. 2B and 3B). This dose-response curve agrees well with that observed for the effect of repeated injections of nicotine on adrenal medullary tyrosine hydroxylase gene transcription rate (compare with Fig. 2A). The nicotine-mediated increase in adrenal TH-mRNA levels was first observed at 3 hr after the initial injection of nicotine and persisted for at least 6 hr after the initial injection (Fig. 4A). Adrenal TH-mRNA levels returned to those observed in saline-treated rats at 24 and 48 hr (Fig. 4A).

Effect of nicotine on adrenal tyrosine hydroxylase protein and enzymatic activity. A single injection of nicotine (3.3 mg/kg) was associated with a 2-fold increase in tyrosine hydroxylase enzymatic activity (saline-treated rats, 0.14 ± 0.02; nicotine-treated rats, 0.28 ± 0.03 for three to five

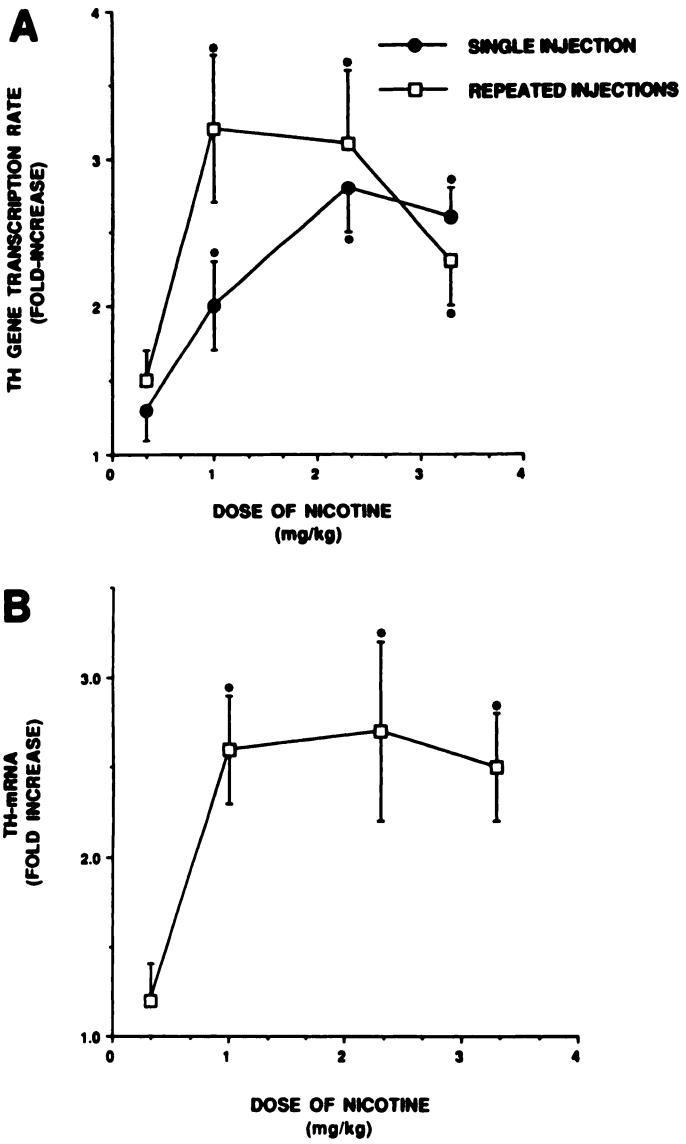

Fig. 2. Dose-response curves for the effects of nicotine on adrenal tyrosine hydroxylase gene transcription rate and TH-mRNA levels. Rats were injected with saline or the following doses of nicotine (subcutaneously): 0.33, 1.0, 2.3, and 3.3 mg/kg. Adrenal glands were removed under sodium pentobarbital anesthesia 20 min after either a single injection or seven injections (once every 30 min for 3 hr) of nicotine or saline. A, The transcription rates in the nicotine-treated rats were expressed as fold increases over those observed in the saline-treated rats. The results were presented in this manner because the tyrosine hydroxylase gene transcription rates in the control animals differed significantly ($p < 0.05$), depending on whether the animals were injected once or repeatedly with saline. The transcription rate values in the rats injected with saline were as follows: single injection, 166 ± 26 ($n = 5$); repeated injections, 96 ± 9 ($n = 8$). The results for the nicotine-treated rats represent the means ± standard errors obtained from five to seven rats. B, Total cellular RNA was isolated from adrenal glands, and TH-mRNA was measured by dot blot hybridization. A single injection of nicotine did not affect adrenal TH-mRNA levels; thus, the data presented in the figure were obtained from rats injected seven times with nicotine or saline. The results represent the means ± standard errors obtained from three rats. *, $p < 0.05$, compared with saline-treated controls.

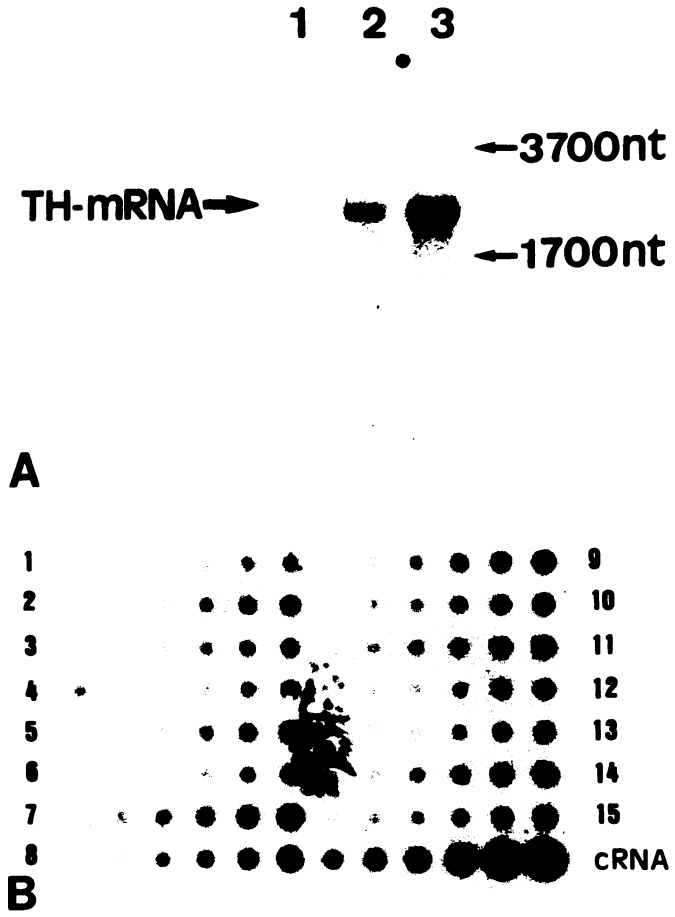

Fig. 3. Northern blot and dot hybridization analyses of adrenal TH-mRNA isolated from saline-treated and nicotine-treated rats. Rats were injected subcutaneously seven times (once every 30 min) with saline or different doses of nicotine, and adrenal glands were removed under anesthesia 3 hr after the initial injection. A, Northern blot analysis was performed by electrophoresis of 8 μg of total cellular RNA through a 1.2% formaldehyde-agarose gel. After transfer to nitrocellulose and hybridization to the radiolabeled probe, autoradiography was performed for 7 days at −85°. *Lanes 1, 2,* and *3*, RNA isolated from rats treated with saline, 0.33 mg/kg nicotine, and 2.3 mg/kg nicotine, respectively. B, Dot blot hybridizations were performed by spotting different amounts (0.2–10 μg) of total cellular RNA onto nitrocellulose. Each row of spots in the figure contains RNA from two different animals; each animal is represented by six dots. The largest amounts of RNA were spotted in the 6th and 12th dots, and then 1/1 serial dilutions were made, moving from the *right* to the *left*. The RNA samples, as designated by the *numbers on the left and right*, are as follows: *1–3*, saline-treated rats; *4–6*, rats treated with 0.33 mg/kg nicotine; *7–9*, rats treated with 1.0 mg/kg nicotine; *10–12*, rats treated with 2.3 mg/kg nicotine; *13–15*, rats treated with 3.3 mg/kg nicotine; and *16*, sense strand RNA standard containing 1 ng of sense strand in the largest dot. The sense strand densities were used as a standard curve to which the sample densities were normalized. The data were then expressed as pg of TH-mRNA sense strand equivalents/μg of total cellular RNA.

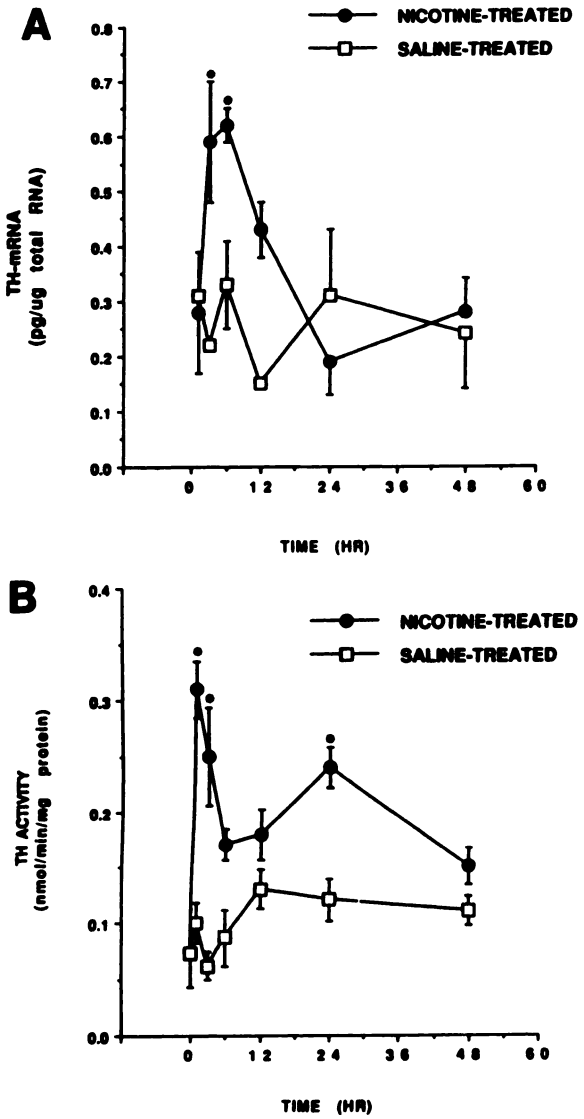

Fig. 4. Time courses of the effect of nicotine on adrenal TH-mRNA levels and tyrosine hydroxylase activity. Rats were injected subcutaneously with either saline or 2.3 mg/kg nicotine seven times (once every 30 min for 3 hr), and adrenal glands were removed at different times after the initial injection. One adrenal gland was used to measure TH-mRNA levels, whereas the other adrenal gland was used to measure tyrosine hydroxylase activity. A, TH-mRNA levels were measured by dot blot hybridization and expressed as the pg of cRNA sense strand/μg of total adrenal RNA. The results represent the means ± standard errors from three or four rats. B, Tyrosine hydroxylase activity was measured using 0.2 mM 6-methyl-5,6,7,8-tetrahydropterine and expressed as nmol of product formed/min/mg of protein. The results represent the means ± standard errors from five to seven animals. *, $p < 0.05$, compared with saline-treated controls.

animals, $p < 0.01$ by two-tailed Student's t test) at 1 hr after injection, but enzyme activity was identical to that observed in saline-treated rats at 3 hr after injection and at all later time points tested (6, 12, 24, and 48 hr). This rapid but transient increase in enzyme activity was presumably due to activation of preexisting enzyme molecules.

When rats were injected repeatedly (once every 30 min for 3 hr) with 3.3 mg/kg nicotine, tyrosine hydroxylase activity increased 3–4-fold at 1 and 3 hr after the initial injection of the drug (Fig. 4B). This elevation of enzyme activity diminished at 6 and 12 hr. However, a 2-fold increase in enzyme activity was observed at 24 hr after the initial injection; enzyme activity returned to control levels at 48 hr. Western blot analysis was used to determine whether the increased activity observed at 24 hr was due to elevated enzyme protein. Fig. 5 presents a representative Western blot. The tyrosine hydroxylase antiserum recognized two adrenal supernatant proteins, possessing apparent molecular masses of approximately 120 and 60 kDa. The 60-kDa protein comigrated with purified tyrosine hydroxylase and was not observed using antiserum for the Western blot that was preabsorbed with an excess of purified tyrosine

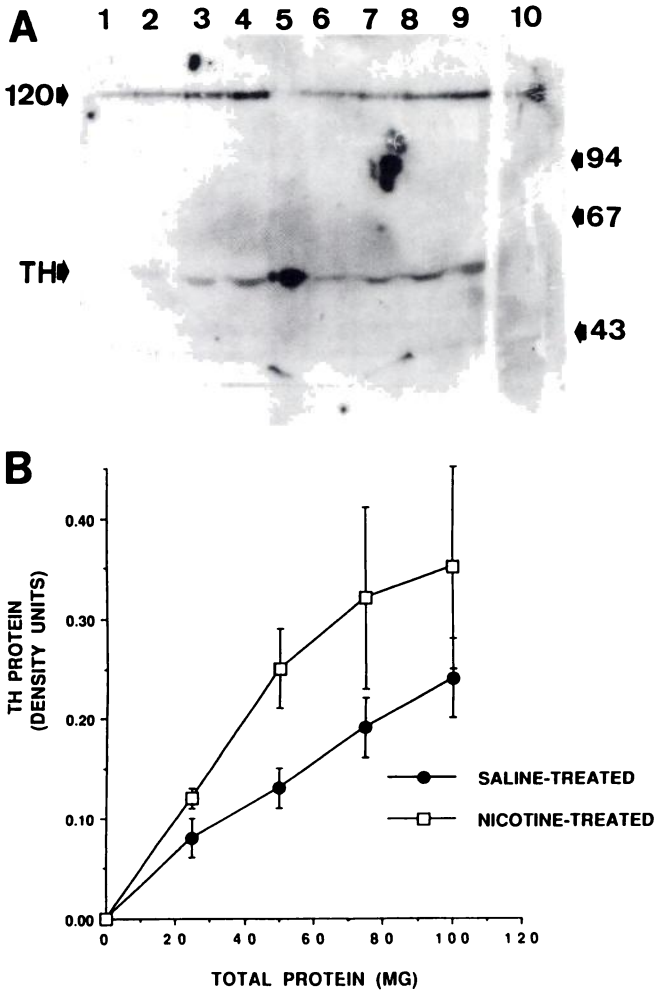

Fig. 5. Western blot analysis of tyrosine hydroxylase protein isolated from rat adrenal glands. Rats were injected subcutaneously with either saline or 2.3 mg/kg nicotine seven times (once every 30 min), and adrenal glands were removed under sodium pentobarbital anesthesia 24 hr after the first injection. Adrenal supernatants were isolated, and different volumes of supernatant containing 25–100 μg of protein were subjected to electrophoresis on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose and subjected to Western analysis using tyrosine hydroxylase antiserum and radiolabeled Protein A. Autoradiography was performed for 1–3 days at −85°. A, *Lanes 1–4* and *6–9*, adrenal supernatant protein from saline-treated and nicotine-treated rats, respectively. *Lanes 1* and *6*, 25 μg of protein; *lanes 2* and *7*, 50 μg of protein; *lanes 3* and *8*, 75 μg of protein; *lanes 4* and *9*, 100 μg of protein. *Lane 5*, purified tyrosine hydroxylase. *Lane 10*, adrenal supernatant protein (75 μg) that was analyzed using antiserum that had been preabsorbed with purified tyrosine hydroxylase. *Arrows on the left*, positions of the tyrosine hydroxylase band and the unknown protein band that interacts with this antiserum but is not related to tyrosine hydroxylase. *Numbers on the right*, positions of standard proteins used to determine molecular weight. B, The density units of each adrenal tyrosine hydroxylase protein band were divided by the density units of the band corresponding to purified tyrosine hydroxylase on the same autoradiogram. The normalized density units were plotted against the μg of protein that were loaded into the appropriate lanes of the gel. Each point represents the mean ± standard error from six experiments.

hydroxylase. These results indicated that the 60-kDa adrenal protein was tyrosine hydroxylase; the identity of the other protein was not investigated. The fold increases (2-fold), measured 24 hr after drug treatment, in tyrosine hydroxylase protein estimated by this Western analysis and in tyrosine hydroxylase

TABLE 2

Effect of nicotine on tyrosine hydroxylase (TH) protein and enzymatic activity

Rats were injected subcutaneously with either saline or 2.3 mg/kg nicotine seven times (once every 30 min for 3 hr). Adrenal glands were removed under anesthesia 24 hr after the initial injection. The results represent the means ± standard errors obtained for five or six animals. The data were analyzed by the Student's two-tailed *t* test.

Treatment	TH protein	TH enzyme activity
	units/μg protein	*mmol/min/mg protein*
Saline	2.5 ± 0.3	0.12 ± 0.02
Nicotine	4.9 ± 0.8[a]	0.24 ± 0.02[a]

[a] $p < 0.05$, compared with saline-treated controls.

TABLE 3

Effect of cholinergic receptor antagonists on the nicotine-mediated stimulation of adrenal medullary tyrosine hydroxylase (TH) gene transcription rate

Rats were injected intraperitoneally with either 15 mg/kg hexamethonium, 15 mg/kg mecamylamine, or 30 mg/kg atropine, 1 hr and 3–5 min before the subcutaneous injection of saline or 2.3 mg/kg nicotine, and adrenal glands were removed 20 min after the last injection. Results are expressed as the means ± standard errors from the number of rats in parentheses.

Pretreatment	Relative TH gene transcription rate	
	Saline	Nicotine
	ppm	
None (8)	170 ± 18	435 ± 29[a]
Hexamethonium (8)	206 ± 37	344 ± 29[a,b]
Mecamylamine (6)	220 ± 30	303 ± 33[a,b]
Atropine (3)	222 ± 54	381 ± 35[a]

[a] $p < 0.05$, compared with saline-treated controls.
[b] $p < 0.05$, compared with nicotine-treated rats that were not pretreated with antagonists.

enzyme activity were approximately equivalent (Table 2). The nicotine-mediated increases in tyrosine hydroxylase enzyme were also equivalent to the preceding nicotine-mediated increases in tyrosine hydroxylase gene transcription rate and TH-mRNA levels.

Effect of nicotinic receptor antagonists on the nicotine-mediated elevation of the tyrosine hydroxylase gene transcription rate in rat adrenal medulla. In order to determine whether the activation of the adrenal tyrosine hydroxylase gene by nicotine was due to stimulation of nicotinic receptors, rats were treated with the nicotinic receptor blockers hexamethonium (15 mg/kg) or mecamylamine (15 mg/kg), 1 hr and 3–5 min before a single injection of saline or nicotine (2.3 mg/kg). Hexamethonium by itself did not significantly affect the transcription rate (Table 3). Hexamethonium pretreatment inhibited the nicotine-mediated stimulation of the adrenal medullary tyrosine hydroxylase gene transcription rate by 30–40% (Table 3). Even in the presence of hexamethonium, nicotine administration elevated the transcription rate 2-fold over that observed in saline-treated animals. Similarly, mecamylamine treatment did not significantly affect rat adrenal tyrosine hydroxylase gene transcription rate, but it blocked the nicotine-mediated effect by approximately 50% (Table 3). The doses of nicotinic receptor blockers used in this study completely inhibited the nicotine-mediated activation of tyrosine hydroxylase in denervated rat adrenal glands.[2] Furthermore, lower doses of these blockers inhibited by >90% the elevation of cyclic AMP elicited by carbachol in the rat adrenal medulla (32).

[2] Fossom, L. H., C. Sterling, and A. W. Tank. Activation of tyrosine hydroxylase by nicotine in rat adrenal medulla. *J. Neurchem.*, in press.

Effects of other drugs on rat adrenal medullary tyrosine hydroxylase gene transcription rate. The effects of a number of other cholinergic agonists and antagonists on tyrosine hydroxylase gene transcription in rat adrenal medulla were investigated. Treatment of the rats with a high dose (30 mg/kg) of the muscarinic cholinergic receptor antagonist atropine did not significantly affect tyrosine hydroxylase gene transcription, nor did it block the stimulation of the gene by nicotine (Table 3). The cholinergic agonist carbachol, which stimulates both nicotinic and muscarinic receptors, elicited a 2–3-fold elevation of tyrosine hydroxylase gene transcription rate (Table 4). This elevation was not blocked by hexamethonium, but it was inhibited by atropine (Table 4). Bethanechol, a muscarinic receptor agonist, produced a 2–3-fold elevation of tyrosine hydroxylase gene transcription rate (Table 5).

We also tested the effects of two noncholinergic drugs on the tyrosine hydroxylase gene transcription rate in rat adrenal medulla. Reserpine induces both tyrosine hydroxylase enzyme and TH-mRNA in rat adrenal medulla (4, 5, 7). As expected, reserpine also stimulated the tyrosine hydroxylase gene, when measured 3 hr after its injection (Table 5). The convulsant drug picrotoxin was administered to determine whether convulsant activity leads to activation of the adrenal medullary tyrosine hydroxylase gene. This drug elicited convulsions that started 3–5 min after injection and persisted throughout the entire 20 min before euthanasia. This treatment elicited a 2-fold stimulation of the gene in the adrenal medulla (Table 5).

TABLE 4

Effect of carbachol on adrenal tyrosine hydroxylase gene transcription rate

Rats were injected intraperitoneally with saline or 1 mg/kg carbachol, and adrenal glands were removed under sodium pentobarbital anesthesia 20 min after injection. Hexamethonium (15 mg/kg) and atropine (30 mg/kg) were injected intraperitoneally 1 hr and 3–5 min before the injection of saline or carbachol. The results represent the means ± standard errors obtained from five or six rats.

Pretreatment	Relative transcription rate	
	Saline	Carbachol
	ppm	
None	166 ± 16	371 ± 56[a]
Hexamethonium	247 ± 38	470 ± 84[a]
Atropine	246 ± 29	214 ± 34[b]

[a] $p < 0.05$, compared with saline-treated controls.
[b] $p < 0.05$ compared with carbachol-treated rats that were not pretreated with an antagonist.

TABLE 5

Effects of different drugs on rat adrenal medullary tyrosine hydroxylase gene transcription rate

Rats were injected with saline or the following drugs: bethanechol, 10 mg/kg (intraperitoneally); picrotoxin, 2 mg/kg (intraperitoneally); 20% ascorbic acid (subcutaneously); or reserpine, 10 mg/kg dissolved in 20% ascorbic acid (subcutaneously). Adrenal glands were removed under sodium pentobarbital anesthesia 20 min after the injection of saline, bethanechol, or picrotoxin or 3 hr after the injection of ascorbic acid or reserpine. Results are expressed as the means ± standard errors from the number of rats designated in parentheses.

Treatment	Relative transcription rate
	ppm
Saline (7)	184 ± 12
Bethanechol (6)	512 ± 64[a]
Picrotoxin (3)	408 ± 66[a]
Ascorbate (3)	166 ± 15
Reserpine (5)	291 ± 34[b]

[a] $p < 0.05$, compared with saline-treated controls.
[b] $p < 0.05$, compared with ascorbate-treated controls.

Discussion

Numerous studies have demonstrated that tyrosine hydroxylase is induced in the rat adrenal medulla by stimuli that activate the sympathetic nervous system or by drugs that interact with cholinergic receptors on adrenal chromaffin cells (1, 8). More recent studies have shown that the elevation of adrenal tyrosine hydroxylase enzyme protein is preceded by an increase in adrenal TH-mRNA levels (5, 7). In this report, we demonstrate that the systemic administration of nicotine results in the induction of tyrosine hydroxylase enzyme and TH-mRNA in rat adrenal gland. Furthermore, we provide direct evidence that this nicotine-mediated induction of tyrosine hydroxylase is primarily due to an increase in the transcription rate of the tyrosine hydroxylase gene.

In order to perform these studies, we developed a nuclear run-on assay to measure tyrosine hydroxylase gene transcription rate in the rat adrenal medulla. Nuclei isolated from two adrenal medullae contain approximately 15–20 μg of DNA (16 ± 1 μg; 14 rats). Even though there is some variability between experiments, the rate of incorporation of radiolabeled UTP into total RNA in these isolated nuclei ranges from 10 to 30 (19 ± 2; 13 rats) pmol of [α-^{32}P]UTP (600 Ci/mmol)/mg of DNA. Hence, using approximately 0.2 mCi of [α-^{32}P]UTP, it is possible to synthesize and isolate 0.25–0.5 × 10^6 cpm of RNA from nuclei derived from the adrenal medullae of a single rat. This amount of radiolabeled nuclear RNA is more than adequate for measuring radiolabeled tyrosine hydroxylase gene-specific transcripts by hybridization to filters containing the pTHg6.3 genomic probe. Furthermore, the hybridization of nuclear RNA to filter-bound pTHg6.3 is specific for TH-RNA transcripts. This conclusion is evident because, even though the actin gene transcription rate is similar in nuclei isolated from both adrenal medulla and adrenal cortex, very low tyrosine hydroxylase gene-specific signals are measured in nuclei isolated from adrenal cortex, a tissue that normally does not transcribe the tyrosine hydroxylase gene. In addition, RNase treatment of the hybrids formed between pTHg6.3 DNA and radiolabeled RNA from adrenal medullary nuclei produces only a 30–40% decrease in radiolabeled RNA annealed to the probe. This loss is presumably due to the degradation of TH-RNA transcripts that contain sequences present in the 3′ region of the gene (a region that is not included in pTHg6.3). In contrast, RNase treatment of hybrids formed between pTHg6.3 and radiolabeled RNA from adrenal cortex results in the loss of annealed radiolabeled RNA to negligible levels.

The administration of nicotine is associated with a 2–3-fold increase in the rate of transcription of the tyrosine hydroxylase gene in nuclei from rat adrenal medulla. This effect is not apparently due to a generalized increase in the rate of transcription of most genes in rat adrenal medullary nuclei, because 1) there is no effect of nicotine on the incorporation of radiolabeled UTP into total nuclear RNA during the run-on assay and 2) there is no effect of nicotine on adrenal medullary actin gene transcription rate. Time course studies demonstrate that the effect of nicotine is rapid, being maximal 10 min after injection of the drug, and persists for at least 1 hr. However, by 3 hr after injection of high doses of nicotine, the tyrosine hydroxylase gene transcription rate returns to that observed in saline-treated animals. This rapid but transient activation of the tyrosine hydroxylase gene is not sufficient to elicit the induction of either adrenal TH-mRNA or tyrosine hydroxylase en-

zyme. In contrast, when nicotine is repeatedly injected once every 30 min for 3 hr, the tyrosine hydroxylase gene transcription rate remains elevated for at least 3 hr. This prolonged activation of the gene leads to the subsequent induction of TH-mRNA and tyrosine hydroxylase enzyme. In an earlier study, Otten *et al.* (12) showed that a 2–4-hr duration of stress is required to observe a significant induction of adrenal tyrosine hydroxylase. Our results explain this observation, because a 3-hr elevation of tyrosine hydroxylase gene transcription is necessary to induce the enzyme.

Dose-response analysis demonstrates that the adrenal tyrosine hydroxylase gene is activated using relatively high doses of nicotine. The two highest doses employed in this study (2.3 and 3.3 mg/kg) elicit convulsions. Masserano *et al.* (33) have shown that convulsive activity associated with electroconvulsive shock results in the induction of adrenal tyrosine hydroxylase; hence, it is possible that the convulsions elicited by nicotine are the cause of the stimulation of the tyrosine hydroxylase gene. The results demonstrating that picrotoxin, a convulsant agent, also activates the adrenal medullary tyrosine hydroxylase gene support this idea. However, the effect of nicotine on adrenal tyrosine hydroxylase gene transcription cannot be totally explained by the associated convulsions. A 1.0 mg/kg dose of nicotine does not produce convulsions in any of the rats, yet this dose activates the gene 2–3-fold when injected once or repeatedly for 3 hr. Furthermore, when 2.3 or 3.3 mg/kg nicotine is administered repeatedly, convulsions are not observed after the first injection. Hence, as observed by other workers (34), tolerance to the convulsive effect of nicotine develops rapidly. However, after 3 hr of repeated injections of these high doses, the tyrosine hydroxylase gene is still activated. This persistent activation of the gene cannot be explained by convulsive activity, because the transcription rate after a single injection of nicotine (and consequently after a single convulsive episode) is identical to that observed in untreated animals 3 hr after the injection of the drug. Thus, even though convulsive activity may play a role in stimulating adrenal tyrosine hydroxylase gene transcription in rats injected once with high doses (2.3 mg/kg or greater) of nicotine, our results suggest that nicotine can also activate the tyrosine hydroxylase gene in the adrenal medulla by mechanisms that are independent of convulsive activity.

Hexamethonium and mecamylamine inhibit the nicotine-mediated increase in tyrosine hydroxylase gene transcription rate by only 30–50%. This lack of complete inhibition by these nicotinic antagonists may be due to an incomplete blockade of these receptors. However, the doses of these antagonists used in this study have been shown to be effective in blocking adrenal chromaffin cell nicotinic receptors in other studies (32, 35).[2] Hence, our results support the hypothesis that nicotine elevates the rate of transcription of the tyrosine hydroxylase gene in the adrenal medulla by two mechanisms, 1) by directly stimulating nicotinic receptors present on adrenal chromaffin cells and 2) by stimulating central nervous system pathways that result in the stimulation of the splanchnic nerve and the consequent release from splanchnic nerve terminals of acetylcholine and other neurotransmitters that interact with receptors other than the nicotinic cholinergic receptor. We speculate that it is the stimulation of these nonnicotinic receptors (either muscarinic cholinergic or noncholinergic receptors) that leads to the elevation of tyrosine hydroxylase gene transcription rate in the presence of the nicotinic receptor blockers. Alternatively, nicotine elicits increases in circulating corticosteroids in rats (36). Tyrosine hydroxylase is induced by glucocorticoids in rat pheochromocytoma cells (13, 23, 28) and in rat superior cervical ganglion (37, 38). Evidence suggests that increases in circulating glucocorticoids do not induce tyrosine hydroxylase in rat adrenal medulla, presumably due to the presence of adrenal cortex-derived corticosteroids that continually bathe the adrenal medulla. Nevertheless, it is possible that increases in blood corticosteroids may play a role in the regulation of adrenal medullary tyrosine hydroxylase gene transcription rate after nicotine administration.

With respect to the hypothesis that nicotine acts via central mechanisms to stimulate the transcription rate of the tyrosine hydroxylase gene in rat adrenal medulla, the inability of mecamylamine to completely block the effect of nicotine on adrenal tyrosine hydroxylase gene transcription is perplexing; mecamylamine blocks both central and peripheral effects of nicotine (39). This result suggests that nicotine activates the splanchnic nerve (at least partially) by stimulating the central nervous system via mechanisms that do not involve nicotinic receptors. Alternatively, the mecamylamine concentration in the brain may not be high enough under these experimental conditions to inhibit totally the ion channels associated with the central nicotinic receptors.

We have also investigated the effects of a number of other cholinergic agonists on adrenal tyrosine hydroxylase gene transcription rate. Carbachol induces tyrosine hydroxylase in rat adrenal medulla (35). Our results demonstrate that carbachol activates the tyrosine hydroxylase gene in the rat adrenal medulla. Hexamethonium has been reported to block the carbachol-mediated stimulation of cyclic AMP and induction of tyrosine hydroxylase in rat adrenal medulla (35). In contrast, our results demonstrate that hexamethonium does not block the carbachol-mediated activation of the tyrosine hydroxylase gene; however, high doses of atropine dramatically inhibit the effect of carbachol on rat adrenomedullary tyrosine hydroxylase gene transcription rate. Furthermore, the muscarinic agonist bethanechol stimulates the tyrosine hydroxylase gene in rat adrenal medulla. Rat adrenal chromaffin cells possess both nicotinic and muscarinic cholinergic receptors (40). Our results suggest that the immediate effect of carbachol is to stimulate adrenomedullary muscarinic receptors that mediate the regulation of the tyrosine hydroxylase gene. However, further work is needed to verify this apparent muscarinic receptor-mediated regulation of tyrosine hydroxylase gene expression in rat adrenal medulla, because the dose of atropine used in this study is relatively high and because it is not known whether the activation of adrenal medullary muscarinic receptors leads to the induction of TH-mRNA and tyrosine hydroxylase protein. The discrepancy between our results and those reported previously concerning whether carbachol regulates tyrosine hydroxylase gene expression via nicotinic or muscarinic receptors is not readily explained. Because the previous studies measured the effect of carbachol on adrenal tyrosine hydroxylase activity 24 hr after treatment, it is possible that the nicotinic effects of carbachol become more pronounced after a longer drug exposure than that employed in our studies.

Finally, we show that reserpine treatment is associated with the elevation of the transcription rate of the tyrosine hydroxylase gene in rat adrenal medulla. Reserpine treatment is a

classical method used to reflexively stimulate the sympathetic nervous system and consequently induce tyrosine hydroxylase and TH-mRNA in rat adrenal gland. Our results demonstrate directly that this induction is due at least partially to the activation of the tyrosine hydroxylase gene.

In earlier studies, nicotine has been shown to induce tyrosine hydroxylase 3–4-fold in the rat adrenal gland after administration of the drug (3.3 mg/kg) twice daily for 7–14 days (41). In agreement with these results, adrenal TH-mRNA levels also increase 3 days after treatment of the rats with nicotine using this paradigm (10).[3] These results are surprising, because a single injection of nicotine elevates the transcription rate of the tyrosine hydroxylase gene for less than 3 hr, and this transient effect on transcription rate does not lead to an elevation of TH-mRNA. Consequently, the simple model in which tyrosine hydroxylase gene transcription rate must be elevated for at least 3 hr by continual intake of nicotine would not predict that single injections of nicotine twice daily would elevate TH-mRNA levels. Chronic treatment of rats with nicotine may lead to regulation of the tyrosine hydroxylase gene by mechanisms that persistently elevate the gene transcription rate or may result in the activation of mechanisms that increase the stability of TH-mRNA in the adrenal medulla. Further work on the effect of nicotine administration for prolonged periods of time is necessary to answer these questions.

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[3] A. W. Tank, unpublished observations.

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