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Catecholamine Secretory Vesicle Stimulus-Transcription Coupling *in Vivo*

DEMONSTRATION BY A NOVEL TRANSGENIC PROMOTER/PHOTOPROTEIN REPORTER AND INHIBITION OF SECRETION AND TRANSCRIPTION BY THE CHROMOGRANIN A FRAGMENT CATESTATIN*

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Stimulation of chromaffin cell secretion *in vitro* triggers not only secretion but also resynthesis of just released catecholamines and chromogranin A, the precursor of the catecholamine release-inhibitory, nicotinic cholinergic antagonist peptide catestatin. Does stimulus-transcription coupling occur *in vivo*? And does catestatin antagonize secretion and transcription *in vivo*? To answer these questions, we employed a novel mouse strain harboring a chromogranin A promoter/firefly luciferase reporter transgene. Tissue-specific expression of the reporter was established by both luminescence and reverse transcription-PCR. Secretion and transcription *in vivo* were triggered by either direct nicotinic stimulation or vesicular transmitter depletion. Nicotinic blockade *in vivo* was attempted with either the classical antagonist chlorisondamine or the novel antagonist catestatin. Luciferase reporter expression was exquisitely sensitive over a large dynamic range, was specific for the transgenic animals, and paralleled typical neuroendocrine distribution of endogenous chromogranin A. Adrenal ontogeny revealed a rise of embryonic transgene expression until embryonal day 18, with an abrupt postnatal decline. Direct nicotinic stimulation of chromaffin cells caused catecholamine release and transgene transcription, each of which was nearly completely blocked by chlorisondamine. Similar adrenal results were obtained during vesicular catecholamine depletion. Both secretion and transcription were substantially blocked in the adrenal gland by catestatin. In brain and sympathetic nerve, stimulation of transcription was more modest, and reserpine responses were only incompletely blocked by chlorisondamine or catestatin, perhaps because of limited blood-brain barrier penetration by these cationic antagonists. Thus, nicotinic cholinergic stimulus-transcription coupling occurs *in vivo* and can be provoked either directly or indirectly (by vesicular transmitter depletion). Such coupling triggers the biosynthesis of chromogranin A, the precursor of catestatin. Catestatin itself blocks stimulation of both secretion and transcription *in vivo*. Thus, chromogranin A and its catestatin fragment may lie at the nexus of nicotinic cholinergic signaling *in vivo*.

Stimulation of the splanchnic (efferent, preganglionic sympathetic) nerves innervating the adrenal medulla and sympathetic postganglionic axons releases multiple neurotransmitters, including acetylcholine (acting on neuronal nicotinic cholinergic receptors), ATP, and chromogranin A, precursor of the catecholamine release inhibitory peptide "catestatin" (bovine chromogranin $A_{344-364}$) (1).

Chromogranin A, the major soluble protein in the core of amine and peptide hormone and neurotransmitter secretory vesicles (2, 3), plays both intracellular and extracellular roles. Within the catecholamine storage vesicle, chromogranin A plays a necessary role in vesiculogenesis and the ability to conduct regulated catecholamine secretion (4). Its extracellular roles derive from its biologically active proteolytic cleavage fragments (3): the catecholamine release-inhibitory fragment catestatin (bovine chromogranin $A_{344-364}$) (1), the vasodilator vasostatin (bovine chromogranin A_{1-76}) (5), and the dysglycemic peptide pancreastatin (porcine chromogranin $A_{240-288}$) (6).

When secretory stimuli (such as acetylcholine interacting with nicotinic cholinergic receptors) trigger transmitter release from chromaffin cells or sympathetic axons, is the resynthesis of just released transmitters also initiated by the secretory stimulus? We have characterized this process (sometimes called "stimulus-secretion-synthesis coupling" or "stimulus-transcription coupling") in chromaffin cells *in vitro* (7–9) and established transcriptional activation of chromogranin A by nicotinic cholinergic (physiologic pathway) stimulation. The process occurs at the level of transcript initiation (7), requires particular elements in *cis* in the proximal promoter (7), and has well defined signal transduction pathways in *trans* (8, 9). However, whether this nicotinic cholinergic stimulation of chromogranin A occurs *in vivo* is uncertain.

We therefore set out to explore whether chromaffin cell stimulus-transcription coupling (specifically nicotinic cholinergic transcriptional stimulation of chromogranin A) occurs *in vivo*. To test this possibility, we employed a transgenic strain in which a mouse chromogranin A 4.8-kbp proximal promoter drives the expression of firefly luciferase, an extraordinarily sensitive reporter of gene expression (10–12). In the *in vivo* experiments reported here, we used nicotine (mimicking the autonomic ganglionic transmitter acetylcholine) and reserpine (indirect stimulation by vesicular depletion) to evoke cate-

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cholamine release and expression of the transgene in the adrenal gland, sympathetic nerve, and brain.

At the same time, we wished to test whether the catecholamine release-inhibitory fragment catestatin (1) functions *in vivo*. Our previous characterization of catestatin in chromaffin cells *in vitro* (1, 13–16) documented its mechanism as a nicotinic cholinergic antagonist (17), thereby acting as an autocrine, negative feedback inhibitor of catecholamine release (1). However, it is not yet clear whether catestatin inhibits catecholamine release *in vivo* (*i.e.* in an intact organism).

Our *in vivo* findings confirm *in vitro* studies of nicotinic signaling to both catecholamine secretion and gene transcription, documenting the role of catestatin in both processes. The studies also provide a novel *in vivo* photoprobe to investigate stimulus-transcription coupling in experimental cardiovascular disease states.

MATERIALS AND METHODS

Transgenic Promoter/Reporter Construction—The mouse chromogranin A promoter (−4.8 kbp to +42 bp) was identified by cDNA hybridization, restriction-mapped, excised from a genomic cosmid clone as an *Xho*I/*Hin*dIII fragment, and subcloned into the polylinker of the firefly luciferase reporter vector pXP1, as described previously (18, 19). The promoter's cap (transcription initiation) site was established by primer extension, as described (18, 19). Promoter position numbering is according to bases upstream (−) or downstream (+) of the cap site. The translational start codon (ATG) in mouse chromogranin A begins 258 bases downstream of the cap site (18, 19); thus, the promoter fragment used in these experiments is entirely upstream of the amino acid-encoding region of the chromogranin A gene, and the first ATG downstream of the cap site encodes the initial Met of firefly luciferase.

Generation of Transgenic Mice—The mouse chromogranin A promoter/firefly luciferase reporter transgenic mouse was created by first digesting the above pXP1 mouse chromogranin A 4.8-kbp promoter/firefly luciferase reporter construct with *Pvu*I and *Bam*HI, to linearize and excise the promoter/reporter transgene from the vector, and then purifying the linear transgene for microinjection using Sepharose gel separation, CsCl density gradient centrifugation, and dialysis against microinjection buffer (20). The transgene also contains an SV40 polyadenylation signal and SV40 3′-untranslated sequence, containing an SV40 small T intron (splice donor and acceptor sites) (Fig. 1*A*). The male pronucleus of fertilized eggs from the FVB inbred strain (Taconic Farms Inc., Germantown, NY) was used for microinjection of the transgene. The presence of the transgene was evaluated by either Southern blot or polymerase chain reaction. The transgenic animals were then inbred to homozygosity.

mRNA Abundance by RT[1]-PCR—Total RNA was prepared from freshly dissected mouse tissues with the RNeasy minikit (Qiagen, Valencia, CA), followed by RNase-free DNase I (Qiagen) treatment (to eliminate any residual genomic DNA). Integrity of the RNA was confirmed by the appearance of 28 s and 18 S rRNA bands on ethidium bromide-stained gels. RT-PCR was performed using PTC-200 DNA Engine thermal cyclers (MJ Research, Watertown, MA), employing a Qiagen one-step RT-PCR kit and the following primer pairs (NCBI accession numbers): mouse chromogranin A mRNA (NM_007693), forward chromogranin A 256–278 (5′-AAGTGCGTCCTGGAAGTCATCTC-3′) and reverse chromogranin A 859–840 (5′-GCTTGGCTTTTCTGGCTTGC-3′); firefly luciferase mRNA (M15077), forward firefly 603–626 (5′-TACTGGGTTACCTAAGGGTGTGGC-3′) and reverse firefly 1002–982 (5′-TGGAAGATGGAAGCGTTTTGC-3′); mouse cyclophilin mRNA (X52803), forward cyclophilin 232–255 (5′-GTGGTGACTTTACACGCCATAATG-3′) and reverse cyclophilin 488–467 (5′-ATTCCTGGACCCAAAACGCTCC-3′).

First strand cDNA was prepared from 500 ng of total RNA template by reverse transcription (using Omniscript™ and Sensiscript™ reverse transcriptases) at 54 °C for 30 min, followed by PCR. The PCR protocol began with a 95 °C/15-min step (for simultaneous inactivation of the reverse transcriptases and activation of the HotstarTaq™ DNA polymerase), followed by a three-step amplification profile (94 °C denaturing step for 30 s, 55 °C annealing step for 30 s, and 72 °C extension step for 1 min) for 25 cycles, 72 °C for 10 min, and finally holding at 4 °C. The reaction mixture was composed of 10 μl of 5× RT-PCR buffer, 2 μl of dNTP mix (each at 10 mM), 2 μl each of forward and reverse primers (each at 10 μM), 2 μl of RT-PCR enzyme mix, 0.2 μl of RNasin® ribonuclease inhibitor (at 40 units/μl; Promega), and 5–15 μl (depending on the concentration) of the template RNA and RNase-free water to achieve the final desired total volume (50 μl/amplification).

After PCR, the products were visualized/photographed on 1.5% agarose gels by ethidium bromide staining/310-nm UV fluorescence. PCR products were then purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA), and amplification of the correct targets was verified by DNA sequencing.

As a negative control, when RNA was pretreated with RNase A (Qiagen), no product in the RT-PCR assay was detected after gel electrophoresis. As a second negative control, no PCR product was obtained when water was included instead of RNA samples in the reaction mixture.

Drug Treatments and Tissue Harvesting—The drugs used were nicotine (2.5 mg/kg intraperitoneally; Sigma), the vesicular monoamine transporter inhibitor reserpine (2.5 mg/kg intraperitoneally; Sigma), the neuronal nicotinic cholinergic antagonist chlorisondamine (5 mg/kg intraperitoneally; Tocris-Cookson), the novel nicotinic antagonist catestatin (20 nmol/25-g mouse intraperitoneally), or vehicle. Catestatin (bovine chromogranin $A_{344-358}$; RSMRLSFRARGYGFR) was synthesized and then purified to >95% homogeneity by RP-HPLC; the identity

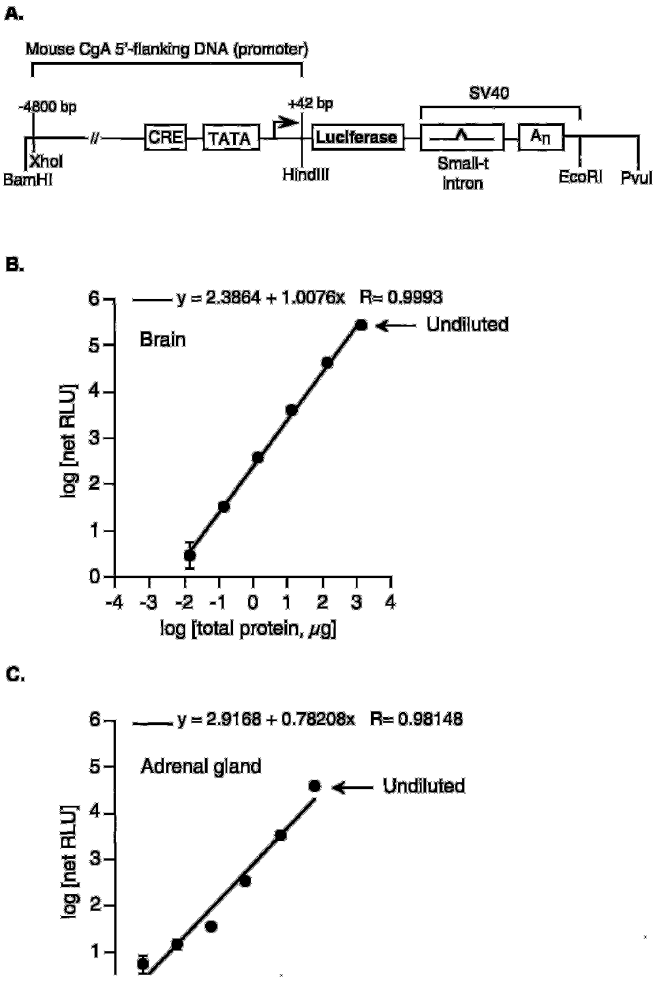

FIG. 1. **The chromogranin A promoter/luciferase reporter transgene.** *A*, structure of the 4.8-kbp mouse chromogranin A promoter/firefly luciferase reporter transgene. The *arrow* indicates the cap (transcription initiation) site. *CRE*, proximal promoter cAMP-response element. *TATA*, proximal promoter TATA box. *SV40*, simian virus 40; A_n, polyadenylation signal. Distances are not drawn to scale. *B* and *C*, sensitivity and dynamic range of the chromogranin A promoter/luciferase reporter transgene assay. Results for brain and adrenal gland homogenates (mean ± S.E.) are shown as $\log_{10}/\log_{10}$ plots. The amount of tissue homogenate protein (in typical 100-μl assay aliquots) is shown on the *horizontal axis*.

[1] The abbreviations used are: RT, reverse transcription; VMAT, vesicular monoamine transporter; HPLC, high pressure liquid chromatography; RLU, relative light unit(s); E*n*, embryonic day *n*; P*n*, postnatal day *n*; F*n*, fetal day *n*.

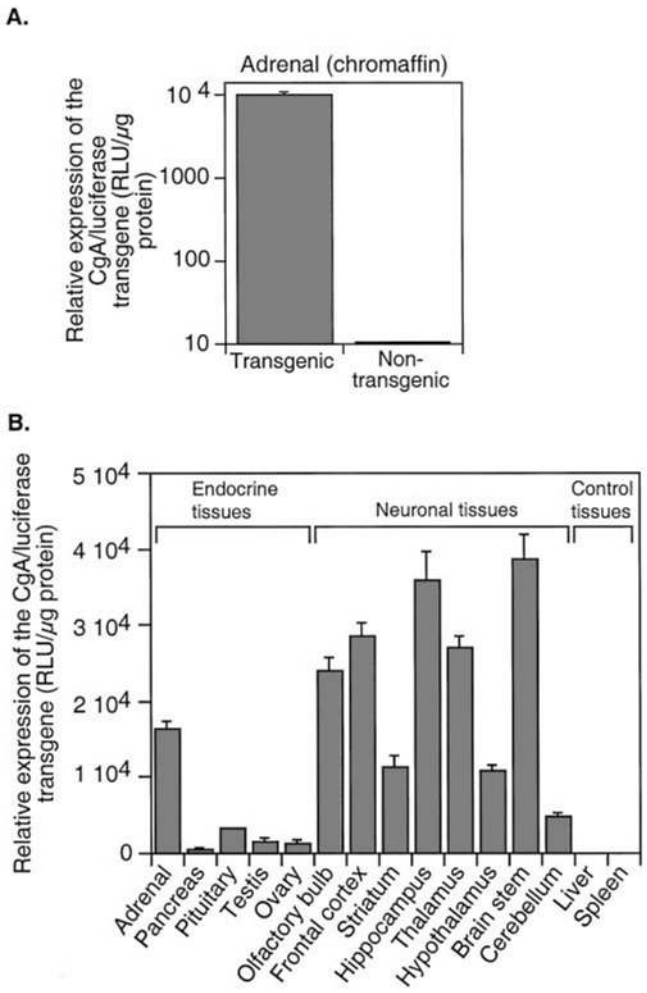

FIG. 2. **Neuroendocrine tissue-specific expression of the chromogranin A promoter/luciferase reporter transgene.** *A*, transgene luciferase expression is specific to mice bearing the construct. Adrenal glands from transgenic and nontransgenic (strain C57BL6) mice were dissected and homogenized in luciferase lysis buffer, and the lysates were assayed in a Berthold luminometer for luciferase activity. Results (mean ± S.E.) are expressed as RLU/μg of protein. $n = 6$ males/group were studied at age 70–90 days. *B*, distribution of transgene expression in mouse tissues: endocrine, neuronal, and control. Results are expressed as RLU/μg of protein. $n = 4$ males/group were studied at age 70–90 days. Ovaries were from $n = 4$ females.

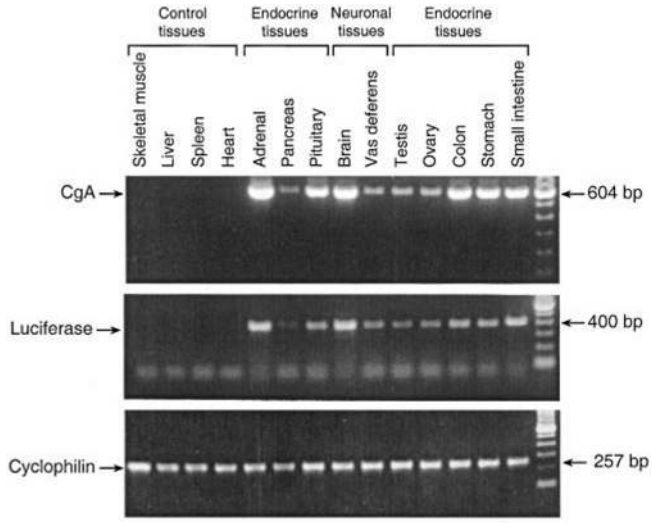

FIG. 3. **Neuroendocrine tissue-specific expression of the chromogranin A promoter: endogenous (chromogranin A) *versus* transgenic (luciferase) mRNAs.** Total RNA was extracted from freshly dissected tissues, and one-step RT-PCR was done using mouse chromogranin A, luciferase, and cyclophilin gene-specific primers (see "Materials and Methods"). First strand cDNA was prepared from total RNA template by reverse transcription (using Omniscript™ and Sensiscript™ reverse transcriptases) followed by PCR. PCR products were purified, and amplification of the correct target genes was verified by DNA sequencing. A representative *image* is shown; *bp* indicates the size of the specific RT-PCR products for each target.

and purity of the final product were verified by re-HPLC and matrix-assisted laser desorption ionization mass spectrometry. The catestatin dose (20 nmol/25-g mouse) was calculated so as to achieve a concentration of ~4 μM in the extracellular space (estimated as 20% of body weight).

In some studies, stimulation of sympathetic outflow was undertaken (directly by nicotine; indirectly by reserpine or vehicle). In dose- and time-dependent studies, reserpine at several doses (1, 5, and 10 mg/kg intraperitoneally) *versus* mock was injected for 4 and 18 h. In other studies, animals were first pretreated with either a nicotinic cholinergic antagonist (chlorisondamine (5 mg/kg intraperitoneally) or catestatin (20 nmol/25 g intraperitoneally)) or vehicle. 30 min later, the sympathetic stimulant was administered: nicotine (2.5 mg/kg intraperitoneally), reserpine (2.5 mg/kg intraperitoneally), or vehicle.

For studies of the effects of the stimulants on plasma catecholamines, mice were treated with drugs (*versus* vehicle) as noted above, and 30 min later they were anesthetized intraperitoneally with a rodent anesthesia mixture (ketamine (60 mg/kg of body weight); xylazine (6.4 mg/kg of body weight); acepromazine (1.2 mg/kg of body weight)). After anesthesia was achieved (1–2 min), blood was then collected from the left ventricle into Eppendorf tubes containing acid/citrate/dextrose as anticoagulant. Blood was kept on ice and centrifuged, and the plasma stored at −70 °C until the assay. Plasma catecholamines were assayed by a sensitive radioenzymatic method (21).

In studies of transcriptional activation of the chromogranin A/luciferase transgene, tissues were obtained from control or drug-treated mice after sacrifice by cervical dislocation, typically 16 h after administration of the drug (nicotine, reserpine, or vehicle) perturbing chromogranin A biosynthesis.

Quantitative Assay of the Chromogranin A/Luciferase Transgene Enzymatic Activity—Freshly dissected tissue samples were collected in 500 μl of ice-cold lysis buffer (0.1 M potassium phosphate buffer, pH 7.8, 1 mM dithiothreitol, 0.1% Triton X-100) and briefly (15–30 s, depending on the volume of the tissue) homogenized by a Tissuemizer homogenizer with a TR-10 power control device (Tekmar, Cincinnati, OH) set at 50% power output. Typical buffer homogenization volumes were 0.25 ml/adrenal gland, and 1.0 ml/brain. The homogenate was centrifuged twice at 14,000 rpm for 30 min at 4 °C, collecting the supernatant in a fresh microcentrifuge tube after the first spin.

100 μl of the clear lysates were then placed in clear plastic polystyrene 12 × 47-mm tubes in an ultrasensitive, low noise luminometer with a 12-watt Peltier-cooled (8 °C) photomultiplier tube, a dark count rate of <100 counts/s (even at 20 °C), spectral sensitivity of 390–620 nm, 20-ns resolution, and quantum efficiency of 24% (AutoLumat LB 953, EG&G Berthold, Bad Wildbad, Germany) with AutoLumat-PC-Control software in a personal computer running DOS on an Intel Celeron RAM chip. 100 μl of the luciferase assay buffer (final concentrations: 100 mM Tris acetate, pH 7.8, 10 mM magnesium acetate, 1 mM EDTA, 0.1 mM luciferin substrate, 3 mM ATP cofactor) were injected to each tube, and flash luminescence was recorded for 10 s and saved in a Kaleidagraph spreadsheet in a personal computer. 3–10 μl (depending on the tissue type) of the tissue lysates were used for total protein measurement (22), by a Coomassie Brilliant Blue dye binding assay reagent (Bio-Rad). Luciferase activities in the various tissues were normalized to protein concentration and expressed as mean relative light units (RLU) ± S.E. One RLU represents 10 pulses released by light quanta from the photon counter. Assay blanks were obtained by measuring luciferase activity in buffer alone (without tissue homogenate).

Data Analysis and Statistics—Data are reported as the mean value ± one S.E. When only two conditions (*e.g.* control and experimental) were compared, the data were evaluated by unpaired *t* tests. When multiple conditions were compared, we used one-way analysis of variance, followed by the Dunnett multiple comparison *post hoc* test, if appropriate. Statistical significance was concluded at $p < 0.05$. Statistics were computed with the programs InStat (GraphPad Software, San Diego, CA), SPSS (Statistical Package for the Social Sciences, Chicago, IL), or Kaleidagraph (Synergy/Abelbeck Software, Reading, PA).

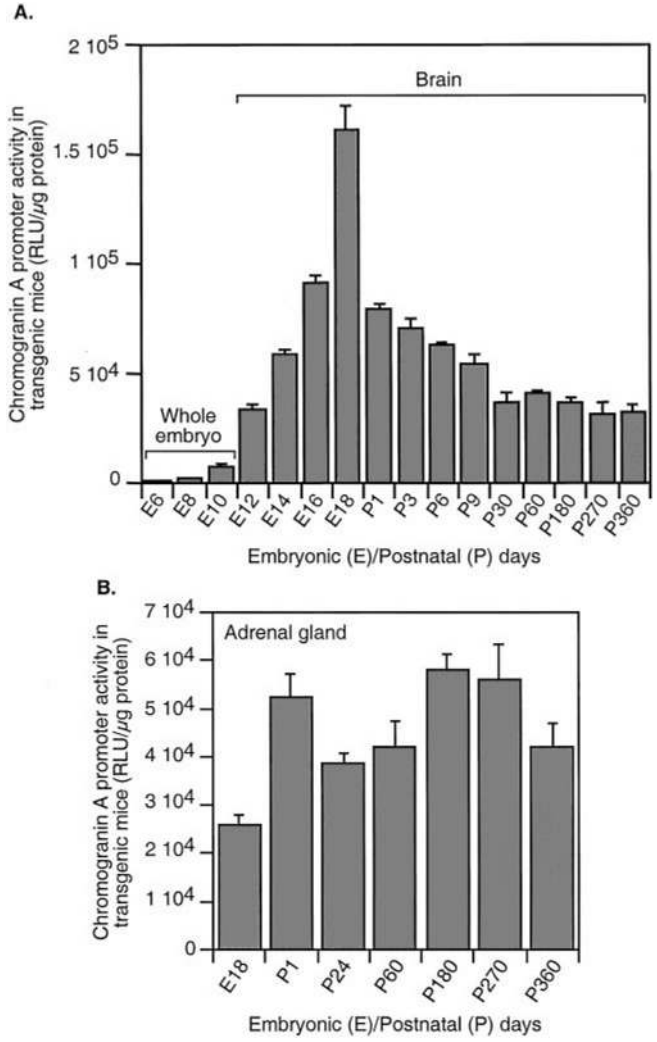

FIG. 4. **Neuroendocrine transgene expression as a function of age.** Ontogenetic and age-dependent expression of the chromogranin A/luciferase transgene. Results (RLU/μg of protein) are expressed as mean ± S.E. $n = 10$ embryonic, postnatal (days P1–P9; both sexes) or $n = 6$ postnatal (days P30–P360; all male) animals were studied at each time point. Embryonic mice were dissected from the uteri of females with established time of conception. *A*, whole embryo/brain. Either the whole embryo (days E6, E8, and E10) or the whole brain (days E12, E14, E16, and E18) was dissected from embryonic mice. The whole brain was dissected from postnatal (days P1–P30) and adult (days P60–P360) mice. *B*, adrenal gland. Adrenal glands were dissected from embryonic (day E18), postnatal (days P1–P24), and adult (days P60–P360) mice.

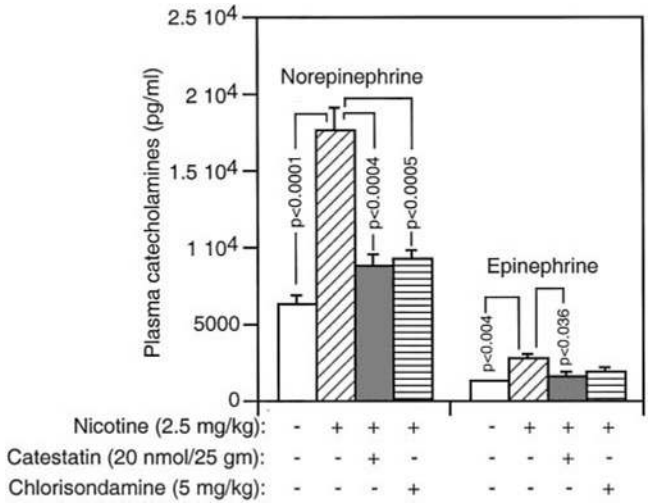

FIG. 5. **Catecholamine release by direct nicotinic cholinergic stimulation and blockade by nicotinic cholinergic antagonists, including catestatin.** The sympathoadrenal system was directly activated by the nicotinic cholinergic agonist nicotine (2.5 mg/kg intraperitoneally) *versus* vehicle (mock). To probe the role of nicotinic cholinergic receptors in secretion after nicotine, animals were pretreated 30 min prior to nicotine with nicotinic cholinergic antagonists, either the classical antagonist chlorisondamine (5 mg/kg intraperitoneally) or the novel antagonist (and chromogranin A fragment) catestatin (20 nmol/25 g intraperitoneally; designed to achieve an extracellular target concentration of ~4 μM), or vehicle (mock). In each experiment, $n = 6$ males were studied, at age 60–70 days. 30 min after nicotine (or vehicle), animals were anesthetized (ketamine, 60 mg/kg of body weight; xylazine, 6.4 mg/kg of body weight; acepromazine, 1.2 mg/kg of body weight), and blood was collected for plasma catecholamine determination (see "Materials and Methods"). Results are shown as mean ± S.E.

RESULTS

Neuroendocrine Tissue-specific Expression of the Chromogranin A Promoter/Luciferase Transgene: Luminescence Enzymatic Activity

The chromogranin A promoter/luciferase reporter transgene (Fig. 1*A*) was an extraordinarily sensitive probe of promoter activity (Fig. 1*B*); the dynamic range of the system extended over 5 $\log_{10}$ orders of magnitude, and tissue samples could be diluted over 10^5-fold (down to nanogram quantities of tissue protein) without reaching the lower limit of detection (Fig. 1*B*).

Assay of chromogranin A/luciferase in the adrenal gland of transgenic mice showed luciferase expression more than ~1000 times background (nonspecific) luminescence in control adrenal glands (Fig. 2*A*).

In agreement with the typical cell type-specific pattern of expression of the endogenous chromogranin A gene (23), we found that the chromogranin A-luciferase transgene was expressed in endocrine (adrenal ≫ pituitary > testis > ovary > pancreas) and neuronal tissues (brain stem, hippocampus, frontal cortex, thalamus, olfactory bulb > striatum, hypothalamus, cerebellum) but not in control tissues (liver, spleen) (Fig. 2*B*).

Tissue Distribution of Transgene and Endogenous Chromogranin A Transcripts: RT-PCR

To directly compare expression of the two forms of the chromogranin A promoter (endogenous gene *versus* transgenic), we employed RT-PCR in the same tissues (Fig. 3).

Consistent with the luminescence data (Fig. 2*B*), both the endogenous chromogranin A gene (chromogranin A mRNA) and the chromogranin A/luciferase transgene (luciferase mRNA) were expressed in endocrine (adrenal > gut > pituitary > testis, ovary) and neuronal tissues (brain > vas deferens). The endogenous and transgenic mRNAs displayed similar rank orders of expression in these neuroendocrine sites. There was no detectable expression of either endogenous or transgenic mRNAs in control tissues (skeletal muscle, liver, spleen, heart). Comparability of mRNA load per lane was confirmed by RT-PCR of a "housekeeping" transcript, cyclophilin.

Expression of the Chromogranin A-Luciferase Transgene during Ontogenetic Development: Brain and Adrenal Gland

Previous *in situ* hybridization studies on the ontogeny (day E16 to P9) of chromogranin A mRNA in rat brain revealed expression in the intermediate cortical layer and dentate gyrus by day E16 (24). In the present study, we detected expression of the chromogranin A/luciferase transgene as early as day E6 (Fig. 4*A*). A steady increase in expression of the transgene in brain was seen up to day E18, with a subsequent abrupt decline after birth (day P1) and then a further slow decline up to day P30, after which expression stabilized up to day P360 (Fig. 4*A*). In contrast,

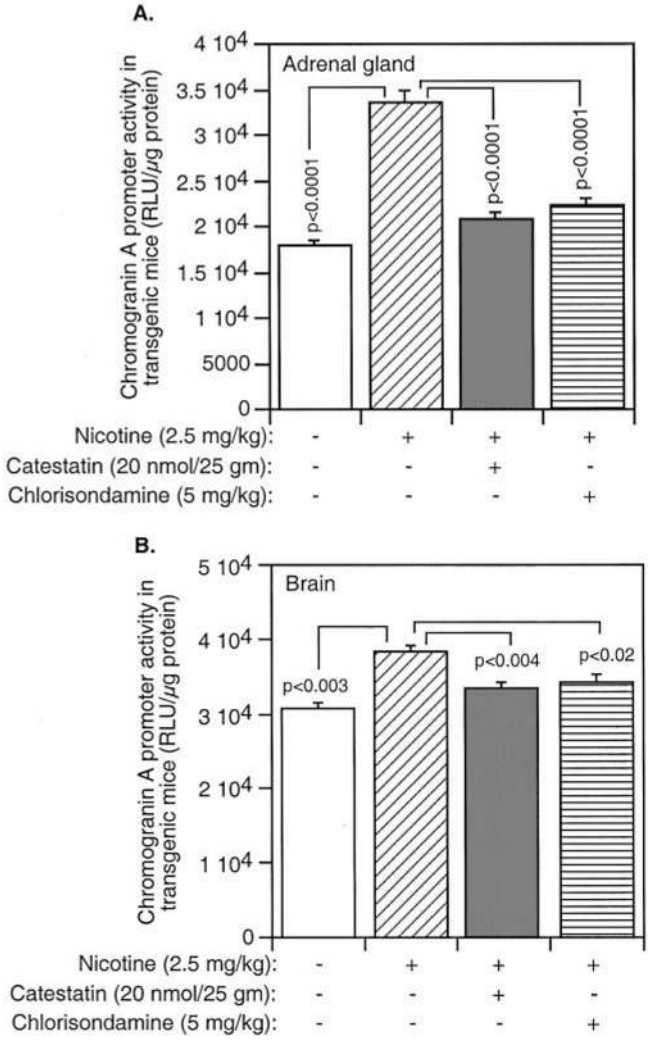

FIG. 6. **Stimulus-transcription coupling after direct nicotinic cholinergic stimulation and blockade by nicotinic cholinergic antagonists, including catestatin.** The sympathoadrenal system was directly activated by the nicotinic cholinergic agonist nicotine (2.5 mg/kg intraperitoneally) *versus* vehicle (mock). To probe the role of nicotinic cholinergic receptors in transcription after nicotine, animals were pretreated 30 min prior to nicotine with nicotinic cholinergic antagonists, either the classical antagonist chlorisondamine (5 mg/kg intraperitoneally) or the novel antagonist (an chromogranin A fragment) catestatin (20 nmol/25 g intraperitoneally; designed to achieve an extracellular target concentration of ~4 μM), or vehicle (mock). In each experiment, $n = 6$ males were studied, at age 60–70 days. 16 h after nicotine (or vehicle), animals were sacrificed to harvest adrenal glands and brains for transgene (chromogranin A promoter/luciferase reporter) and protein assays. The results (mean ± S.E.) obtained in the adrenal glands (*A*) and brains (*B*) were expressed as RLU/μg of protein.

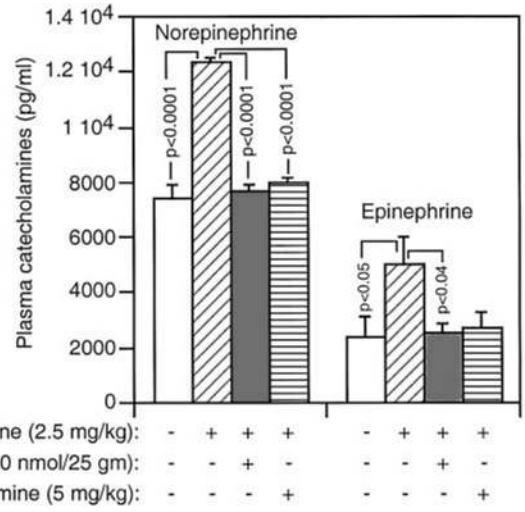

FIG. 7. **Indirect stimulation of the sympathoadrenal system by vesicular transmitter depletion: acute transmitter release and blockade by nicotinic cholinergic antagonists, including catestatin.** The sympathoadrenal system was indirectly activated by the VMAT inhibitor reserpine (2.5 mg/kg intraperitoneally) *versus* vehicle (mock). To probe the role of nicotinic cholinergic receptors in secretion after reserpine, animals were pretreated 30 min prior to reserpine with nicotinic cholinergic antagonists, either the classical antagonist chlorisondamine (5 mg/kg intraperitoneally) or the novel antagonist (and chromogranin A fragment) catestatin (20 nmol/25 g intraperitoneally; designed to achieve an extracellular target concentration of ~4 μM), or vehicle (mock). In each experiment, $n = 6$ males were studied, at age 70–80 days. 30 min after reserpine (or vehicle), animals were anesthetized (ketamine, 60 mg/kg of body weight; xylazine, 6.4 mg/kg of body weight; acepromazine, 1.2 mg/kg of body weight), and blood was collected for plasma catecholamine determination (see "Materials and Methods"). Results are shown as mean ± S.E.

expression of the transgene in the adrenal gland displayed a substantial increment from day E18 to P1 but was followed thereafter by only ~20% fluctuations up to day P360 (Fig. 4*B*).

Direct Nicotinic Cholinergic Stimulation of the Sympathoadrenal System: Acute Transmitter Release, Subsequent Adrenal Stimulus-Transcription Coupling, and Role of Catestatin in Vivo

Acute Transmitter Release—Direct activation of nicotinic cholinergic receptors by nicotine caused acute (30 min) ~2.7-fold release of catecholamines (norepinephrine and epinephrine) from storage vesicles into the bloodstream (Fig. 5). When animals were pretreated with the classical neuronal nicotinic antagonist chlorisondamine, the catecholamine increment was blunted by >80%, confirming specific mediation of the response by nicotinic receptors.

Stimulus-Transcription Coupling—Chromogranin A (25) and its catestatin fragment (14) are co-released by exocytosis with catecholamines. In cultured chromaffin cells *in vitro* (7–9), exocytotic stimuli also program the resynthesis of just released catecholamine storage vesicle contents, a process known as "stimulus-secretion-synthesis coupling" or "stimulus-transcription coupling." Does this phenomenon occur *in vivo*? Treatment with nicotine resulted in a ~2-fold increment in expression of the chromogranin A/luciferase transgene, confirming the phenomenon *in vivo*, and the increment was >80% inhibited by the classical nicotinic cholinergic antagonist chlorisondamine (Fig. 6*A*).

Catestatin in Vivo—The chromogranin A fragment catestatin (1), a novel nicotinic cholinergic antagonist stored and released with catecholamines by exocytosis (14), also blocked the catecholamine secretory response to nicotine by >80% (Fig. 5), indicating that this peptide functions as a nicotinic antagonist upon secretion *in vivo*, extending its role as an autocrine, negative feedback inhibitor of catecholamine release (1). Furthermore, catestatin also blocked the transcriptional response to nicotine by >90% (Fig. 6*A*), establishing an entirely new role for the peptide on gene expression *in vivo*.

Stimulus-Transcription Coupling in Brain after Direct Nicotinic Cholinergic Stimulation

Besides stimulation of neurotransmitter release, nicotine triggers expression of genes encoding enzymes involved in neurotransmitter synthesis such as tyrosine hydroxylase (26), neurotransmitter transporters such as the vesicular acetylcholine transporter (27), neuropeptides such as neuropeptide Y (28), and transcription factors such as c-Fos and the cAMP-response element-binding protein (29).

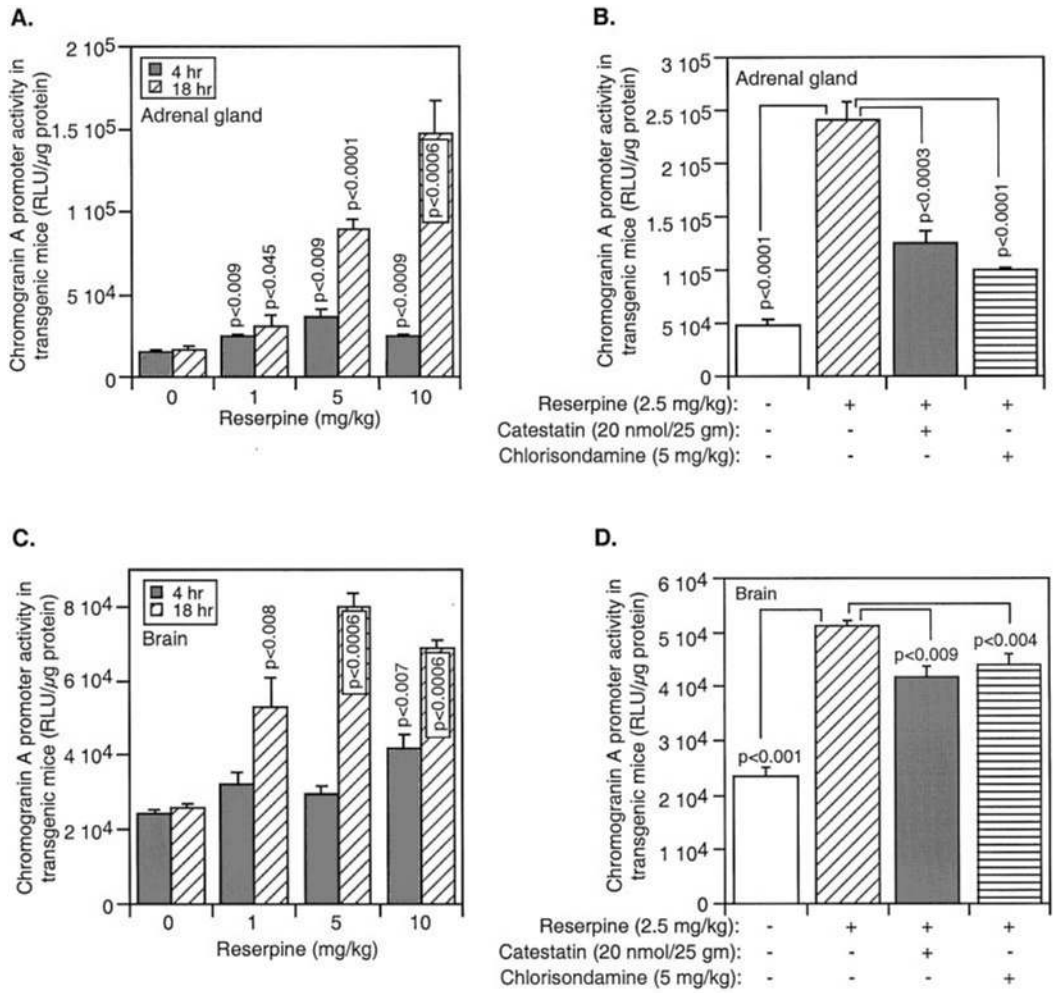

FIG. 8. **Stimulus-transcription coupling after indirect stimulation by vesicular transmitter depletion: time and dose dependence and blockade by nicotinic cholinergic antagonists, including catestatin.** The sympathoadrenal system was indirectly activated by the VMAT inhibitor reserpine (1–10 mg/kg intraperitoneally) *versus* vehicle (mock). To probe the role of nicotinic cholinergic receptors in transcription after reserpine, animals were pretreated 30 min prior to reserpine with nicotinic cholinergic antagonists, either the classical antagonist chlorisondamine (5 mg/kg intraperitoneally) or the novel antagonist (chromogranin A fragment) catestatin (20 nmol/25 g intraperitoneally; designed to achieve an extracellular target concentration of ~4 μM), or vehicle (mock). In each experiment, $n = 6$ males were studied, at age 50–70 days. At the time points shown after reserpine (or vehicle), animals were sacrificed to harvest adrenal glands and brains for transgene (chromogranin A promoter/luciferase reporter) and protein assays. The results (mean ± S.E.) obtained in the adrenal glands (*A* and *B*) and brains (*C* and *D*) were expressed as RLU/μg of protein. *A* and *C*, dose-response and time course relationships over several reserpine doses (0, 1, 5, or 10 mg/kg intraperitoneally) at two time points (4 and 18 h). *B* and *D*, results obtained at one time point (16 h after stimulation), with or without nicotinic cholinergic antagonist pretreatment.

Nicotinic stimulation elevated brain expression of the chromogranin A transgene by ~26% (Fig. 6*B*). This increment was blocked ~70% by the classical nicotinic antagonist chlorisondamine and ~80% by the novel nicotinic antagonist catestatin.

Indirect Stimulation of the Sympathoadrenal System by Vesicular Depletion: Initial Transmitter Release, Subsequent Adrenal Stimulus-Transcription Coupling, and Role of Catestatin in Vivo

Initial Transmitter Release and Catestatin—Vesicular monoamine transporter (VMAT) inhibition by reserpine causes an initial release of stored catecholamines *in vitro* (30) and *in vivo* (31), followed by a prolonged inhibition of catecholamine uptake into storage granules in the adrenal medulla (32) and noradrenergic nerves (33, 34). Treatment with reserpine (Fig. 7) caused acute catecholamine secretion, to plasma levels ~30% greater than basal. Pretreatment with the classical nicotinic cholinergic antagonist chlorisondamine completely blocked the acute secretory response (Fig. 7), suggesting that the acute secretion might be, at least in part, mediated by reflex increments in efferent preganglionic sympathetic (splanchnic) nerve traffic (35, 36).

Stimulus-transcription coupling—We also tested whether initial depletion of stored catecholamines by reserpine, followed by reflex stimulation of efferent sympathetic/splanchnic outflow (35, 36), might also cause stimulation of chromogranin A/luciferase transgene expression. Reserpine caused time- and dose-dependent increments in transgene expression in the adrenal gland (Fig. 8*A*; ~4-fold stimulation at 10 mg/kg). The reserpine-stimulated adrenal transcriptional response was blocked >70% by the nicotinic antagonist chlorisondamine (Fig. 8*B*). Thus, prolonged catecholamine depletion seems to reflexively trigger increments in efferent preganglionic sympathetic (splanchnic) nerve traffic (35, 36).

Catestatin in Vivo—Catestatin not only completely blocked depletion-induced increments of plasma catecholamines (Fig. 7) but also diminished by >60% the chromogranin A transgene activation after reserpine (Fig. 8*B*). Thus, catestatin *in vivo* seems to exert nicotinic cholinergic antagonist activity on both secretory and transcriptional processes in the sympathoadrenal system.

Stimulus-Transcription Coupling in Brain after Indirect Stimulation by Vesicular Depletion

Depletion of neurotransmitter storage by reserpine also increases gene expression of enzymes involved in neurotransmitter synthesis, such as tyrosine hydroxylase (37), as well as neurotransmitter transporters, such as the serotonin transporter (38), and neuropeptides including preproenkephalin (39), preprotachykinin (39), galanin, vasopressin (40), neuropeptide Y (41), chromogranin B, and secretogranin II (40).

In the present study, transmitter depletion caused time- and dose-dependent increments of transgene expression in the brain, with ~3.3-fold stimulation at 5 mg of reserpine/kg (Fig. 8*C*). The increment was blocked ~30% by the classical nicotinic antagonist chlorisondamine and ~35% by the novel nicotinic antagonist catestatin (Fig. 8*D*).

Stimulus-Transcription Coupling in Peripheral Postganglionic Sympathetic Axons (vas Deferens)

The male vas deferens is a rich source of postganglionic sympathetic nerve terminals (25, 31). Contraction of smooth muscle in the vas deferens is elicited by sympathetic nerves co-releasing the neurotransmitters ATP and norepinephrine from large dense core vesicles (42, 43). Reserpine depletes catecholamines from these vesicles (44). Since nicotinic cholinergic receptors are expressed on vas deferens nerve terminals (45), nicotinic cholinergic stimulation should evoke catecholamine and chromogranin A co-release from these same vesicles.

In these studies of the vas deferens (Fig. 9), vesicular depletion by reserpine increased chromogranin A transgene expression by ~1.75-fold, whereas direct nicotinic cholinergic stimulation activated the transgene by ~1.5-fold.

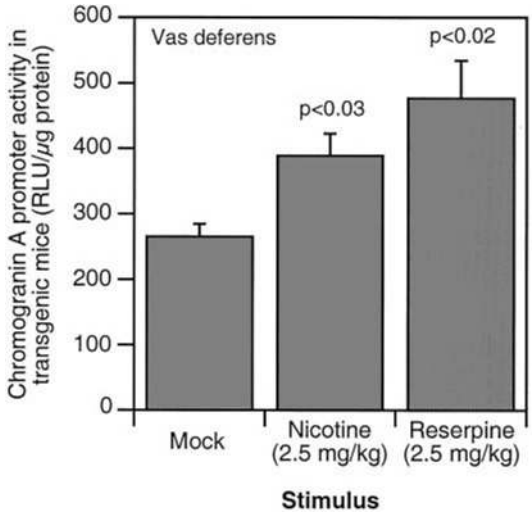

FIG. 9. **Stimulus-transcription coupling in peripheral postganglionic sympathetic axons (vas deferens nerve termini).** $n = 6$ male mice/group, age 60–80 days, were subjected to direct (nicotine, 2.5 mg/kg intraperitoneally) or indirect (vesicular depletion by reserpine, 2.5 mg/kg intraperitoneally) activation of the sympathoadrenal system. Control animals received vehicle (mock). 16 h later, the animals were sacrificed for vas deferens dissection, homogenization, and luciferase assay. Results (RLU/μg of protein) are expressed as mean ± S.E.

DISCUSSION

Neuroendocrine Expression—Specific expression of the chromogranin A promoter/luciferase reporter was verified by its restriction only to mice bearing the transgenic construct (Fig. 2*A*). Consistent with the reported neuroendocrine distribution of chromogranin A (2, 23, 46, 47), we found expression of the chromogranin A/luciferase transgene in endocrine and neuronal tissues but not in control tissues (Fig. 2*B*).

The 4.8-kbp mouse proximal chromogranin A promoter directs correct neuroendocrine expression of the gene in transfected neuroendocrine cells *in vitro* (19, 47). Does this promoter region also direct appropriate expression *in vivo*? The rank orders of endogenous chromogranin A mRNA and transgenic luciferase mRNA expression were across cell types (Fig. 3), indicating that the 4.8-kbp mouse chromogranin A proximal promoter fragment contains information sufficient to direct correct neuroendocrine specific expression *in vivo*.

Detection of the chromogranin A/luciferase transgene in testis, vas deferens, and ovary supports earlier immunohistological findings of endogenous chromogranin A expression in testis (48, 49), vas deferens (50, 51), and ovary (52, 53).

Neuroendocrine Ontogeny of Transgene Expression—Ontogenetic expression of chromogranin A has been explored previously in birds and mammals. In the chick, chromogranin A was detected in the carotid body (day E9) (54) and gizzard (day E12) (55). Chromogranin A mRNA was detected in rat brain at day E16 (24) and in rat enterochromaffin-like cells at day E18 (56). In the fetal pig, chromogranin A immunoreactivity was detected at days F24–F27 in sympathetic ganglia, days F37–F42 in chromaffinoblasts, and days F54–F56 in adrenal medullary cells, declining after day F76 and postnatally (57). In the human adrenal gland, chromogranin A was detected in chromaffin cell precursors by 9 weeks of gestation (58), whereas in the fetal lung chromogranin A was detected as early as 12–14 weeks (59). In the present study, detection of chromogranin A/luciferase transgene expression at day E6 may indicate an especially early role in embryonic development.

Stimulus-Transcription Coupling in Vivo and the Role of Nicotinic Cholinergic Receptors—Nicotine is a powerful stimulant of the sympathoadrenal system, causing release of catecholamines from postganglionic sympathetic neurons and adrenal medulla (60). Nicotine also acts centrally to stimulate dopamine release from nigrostriatal and mesocorticolimbic neurons and norepinephrine release from hippocampus, cerebellum, and locus coeruleus neurons (61–63). In agreement with these findings as well as our previous *in vitro* studies (1, 7), here we documented acute nicotinic stimulation of catecholamine release as reflected by ~2.7-fold increments in plasma catecholamine levels (Fig. 5).

Such nicotinic stimulation in cultured chromaffin cells *in vitro* (7–9) triggers the resynthesis of chromogranin A at the transcriptional level. This process of "stimulus-secretion-synthesis coupling" or "stimulus-transcription coupling" may serve to replete storage vesicles of just released transmitters (7–9).

Enkephalins (64) and catecholamine biosynthetic enzymes (tyrosine hydroxylase, dopamine β-hydroxylase, and phenylethanolamine-*N*-methyltransferase) are up-regulated in response to nicotinic cholinergic stimulation (7, 29, 66, 67). Nicotine can also function centrally to activate tyrosine hydroxylase in the adrenal medulla, both transcriptionally and post-translationally (67). Consistent with our *in vitro* studies of the transfected chromogranin A promoter in chromaffin cells (7–9), we documented ~2-fold direct nicotinic stimulation of chromogranin A/luciferase transgene expression in the adrenal gland (Fig. 6*A*), and the increment was blocked >80% by chlorisondamine. Three conclusions can be drawn: 1) the stimulus-transcription mechanism for repletion of vesicular contents postexocytosis also functions *in vivo*; 2) the stimulus-transcription process is specifically triggered by the nicotinic cholinergic receptor; 3) a 4.8-kbp proximal chromogranin A promoter is sufficient to confer the nicotinic transcriptional response *in vivo*.

Reserpine was also used to explore the stimulus-transcrip-

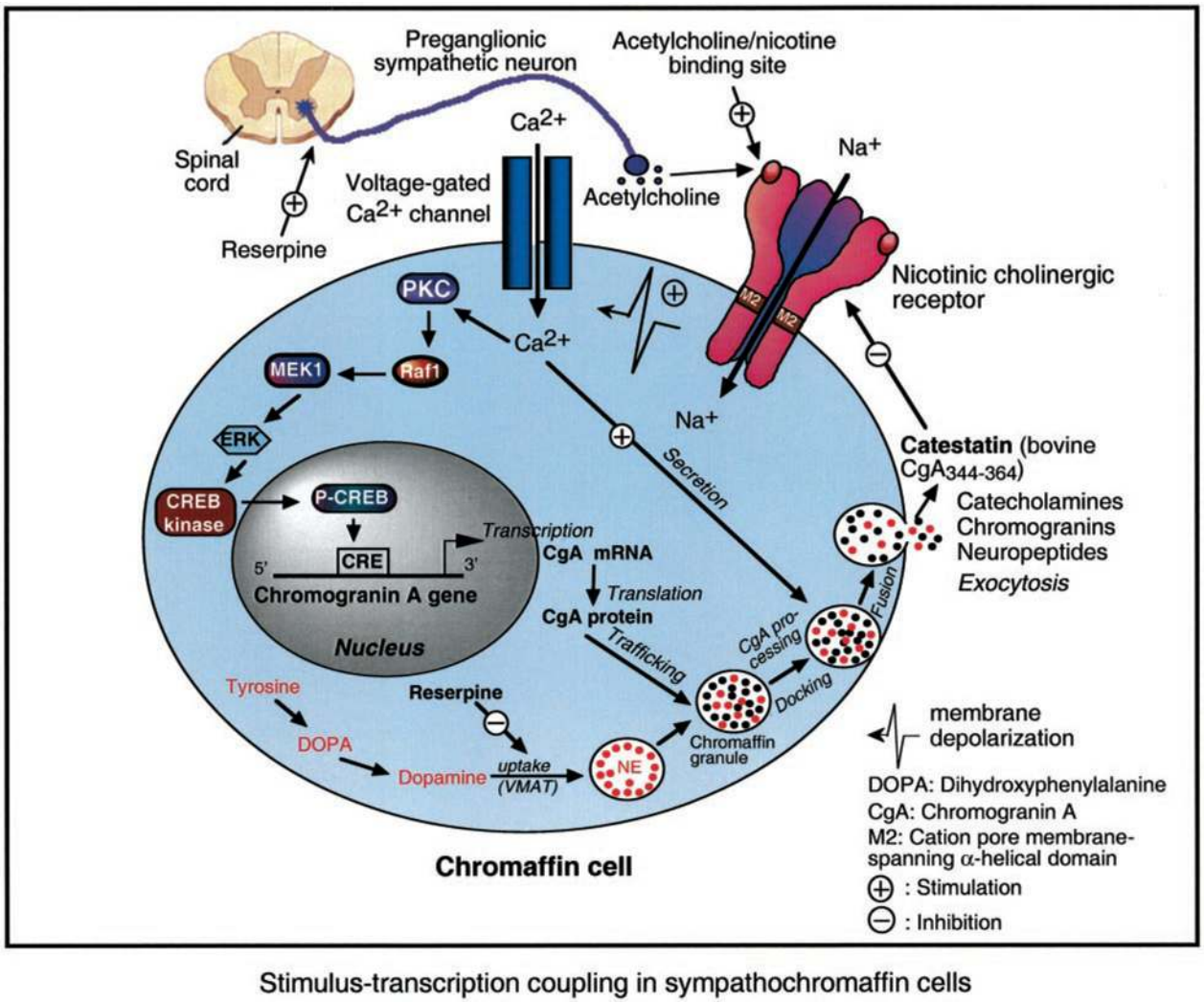

FIG. 10. **Proposed model for the stimulus-transcription coupling to chromogranin A biosynthesis in sympathochromaffin cells and the autocrine inhibitory role of the chromogranin A fragment catestatin.** The nicotinic cholinergic agonist (acetylcholine surrogate) nicotine binds to the nicotinic cholinergic receptor, inducing extracellular Na^+ influx, depolarizing the cell membrane, and causing influx of calcium through voltage-gated calcium channels. Influx of calcium induces both catecholamine release by exocytosis (all-or-none secretion) and chromogranin A gene transcription through a pathway (62–64) involving activation of protein kinase C (*PKC*) and mitogen activated protein kinase (*MAPK*). Catestatin is formed within and secreted from chromaffin granules and inhibits subsequent catecholamine secretion and chromogranin A gene transcription by acting as an endogenous nicotinic cholinergic antagonist. The VMAT inhibitor reserpine impairs (−) catecholamine storage by inhibiting uptake of dopamine or catecholamines into the chromaffin granules, resulting in acute nonexocytotic catecholamine release. By contrast, chronic reflex splanchnic sympathetic activation after catecholamine depletion by reserpine (+) causes the release of acetylcholine, which in turn acts at neuronal/ganglionic nicotinic cholinergic receptors to induce both catecholamine secretion and chromogranin A gene transcription. *NE*, norepinephrine; *ERK*, extracellular signal-regulated kinase; *CRE*, cAMP-response element; *CREB*, cAMP-response element-binding protein.

tion process (Figs. 7–9). The mechanism of action of reserpine involves catecholamine store depletion from sympathetic nerve endings and chromaffin cells (33, 34) through reversible competitive inhibition of VMATs (69). Previous reports revealed that splanchnic denervation diminished reserpine-induced adrenal medullary catecholamine secretion and depletion (70–72), indicating efferent sympathetic neural mediation of reserpine action on chromaffin cell catecholamine release. In agreement with these reports, we found complete blockade of reserpine-stimulated catecholamine secretion by the classical nicotinic cholinergic antagonist chlorisondamine (Fig. 7). >4-fold transcriptional activation of the chromogranin A/luciferase transgene was also inhibited >70% by chlorisondamine, documenting the participation of splanchnic nerve traffic in a reflex response (Fig. 8, *A* and *B*).

Thus, both nicotine and reserpine activate not only acute catecholamine release (Figs. 5 and 7) but also chronic stimulus-transcription coupling (Figs. 6 and 8), albeit by rather different initial mechanisms. Nonetheless, each process involves an obligate role for nicotinic cholinergic receptors: in the case of nicotine itself, a direct role (Figs. 5 and 6), and an indirect, reflex role in splanchnic neurotransmission in the case of vesicular depletion by reserpine (Figs. 7 and 8).

Stimulus-Transcription Coupling in Neurons and Brain—Both nicotine (Figs. 6 and 9) and reserpine (Figs. 8 and 9) provoked increments in chromogranin A/luciferase transgene expression, in both peripheral postganglionic sympathetic axons (Fig. 9) and brain (Figs. 6*B* and 8, *C* and *D*). Reserpine was more powerful than nicotine in stimulating the transgene, whether in the adrenal gland (Fig. 8, *A* and *B*), peripheral noradrenergic nerve terminals (Fig. 9), or brain (Fig. 8, *C* and *D*).

In neurons, the relative degree of transgene activation by either nicotine (~1.26- to ~1.5-fold) (Figs. 6 and 9) or reserpine (~1.75- to ~3.3-fold) (Figs. 8, *C* and D, and 9) was quantitatively similar in brain (Figs. 6*B* and 8, *C* and *D*) and peripheral sympathetic nerves (Fig. 9). However, the degree of luciferase stimulation in neurons was substantially less than the corre-

sponding increment in the adrenal gland after either nicotine (~2-fold) (Fig. 6*A*) or reserpine (~4-fold) (Fig. 8, *A* and *B*). Impaired passage of nicotine or reserpine across the blood-brain barrier (73) cannot easily explain the adrenal *versus* brain/neuron disparity in transgene activation, since vas deferens nerve termini are certainly outside the brain, and both nicotine and reserpine display at least some brain penetration (73), especially over a 16–18-h time course. Reasons for these adrenal *versus* neuronal discrepancies in transgene response are not immediately apparent but might include the well known differences in the subunit composition of nicotinic receptors in the two sites (17) or the marked quantitative differences in catecholamine storage vesicle size, composition, and abundance in chromaffin cells *versus* noradrenergic nerves (25, 31, 74).

The ability of the classical nicotinic cholinergic antagonist chlorisondamine to block stimulus-transcription coupling (Fig. 6) also differed somewhat by site (adrenal *versus* brain) and by stimulus (nicotine *versus* reserpine). In the adrenal gland, both stimuli to transcription (nicotine (Fig. 6*A*) and reserpine (Fig. 8*B*) were blocked >70% by chlorisondamine. By contrast, in brain only nicotine-stimulated transcription (Fig. 6*B*) was substantially (>70%) blocked by chlorisondamine, whereas reserpine-stimulated transcription (Fig. 8*D*) was reduced only ~30% by chlorisondamine. Here we must consider the potential role of the blood-brain barrier in hindering transport of chlorisondamine into the brain; chlorisondamine's two obligate positively charged quaternary amines (*i.e.* bis-quaternary amine structure), coupled with the relatively brief (30-min) period between chlorisondamine pretreatment and subsequent stimulus (nicotine or reserpine) administration, would diminish the probability of effective and timely brain penetration (73) for central nicotinic cholinergic blockade. Indeed, other investigators (68, 75) have found that effective central nervous system nicotinic cholinergic blockade by parenteral chlorisondamine may require larger doses or longer periods of time (as long as 12–21 days of pretreatment).

Role of Catestatin in Vivo—The chromogranin A biologically active fragment catestatin (*e.g.* bovine chromogranin $A_{344-364}$) is proteolytically cleaved from chromogranin A in catecholamine storage vesicles *in vivo* (14), whereupon it can be co-released by exocytosis along with catecholamines (14). It then acts as a nicotinic cholinergic antagonist (17) to block catecholamine release in response to the physiological trigger, acetylcholine (1). Whereas catestatin's nicotinic cholinergic blocking actions have been extensively characterized in cultured chromaffin cells *in vitro* (1, 13–16), its potential actions *in vivo* have not been extensively explored.

Here we found that catestatin *in vivo* effectively blocked not only the catecholamine secretory responses (nicotine (Fig. 5) and reserpine (Fig. 7)) but also the adrenal transcriptional responses (nicotine (Fig. 6) and reserpine (Fig. 8)) to nicotinic cholinergic-mediated stimuli. In each case (secretion or transcription), blockade by catestatin was comparable in magnitude with blockade achieved by the classical nicotinic cholinergic antagonist chlorisondamine. In one setting (reserpine-induced brain transcription) (Fig. 8*D*), catestatin and chlorisondamine were only partially (~30–35%) effective in blocking stimulus-induced transcription. In this setting, it is noteworthy that both chlorisondamine and catestatin are predicted to exhibit poor penetration of the blood-brain barrier (73) (chlorisondamine because of its two obligate quaternary amine moieties (*i.e.* bis-quaternary amine structure) and catestatin (as used here: bovine chromogranin $A_{344-358}$; RSMRLSFRARGYGFR) because of its highly cationic nature (calculated pI of ~12.7, with 33% Arg content) and relatively high molecular weight (1861 g/mol) for a drug). Finally, catestatin is a somewhat less potent inhibitor of catecholamine release from neurites than chromaffin cells (1).

Conclusion and Perspectives—Thus, chromogranin A (and its catestatin fragment) seem to be at the virtual nexus of nicotinic cholinergic signaling to both secretion and transcription *in vivo*. The catecholamine release-inhibitory peptide catestatin seems clearly to function *in vivo* as a nicotinic cholinergic antagonist, blocking agonist-induced increments in both secretion (Figs. 5 and 7) and transcription (Figs. 6 and 8, *B* and *D*) in sympathochromaffin cells. These actions of exogenous catestatin clarify and put into functional perspective recent observations in humans on the inferred actions of endogenous catestatin *in vivo* (65), in which we observed that a diminution of plasma catestatin was associated with augmented risk of developing hypertension and increased pressor responses to environmental stressors.

These *in vivo* findings extend the significance of our previous *in vitro* studies of nicotinic signaling to catecholamine secretion and gene transcription in chromaffin cells (1, 13–16), establish a fundamental role for chromogranin A and its catestatin fragment at the nexus of nicotinic cholinergic signaling (Fig. 10), and provide a sensitive, novel *in vivo* photoprobe to investigate stimulus-transcription coupling in experimental cardiovascular disease states.

Fig. 10 presents a diagram summarizing our principal findings and conclusions. Although simply a model, it does present a framework for integrating the results and formulating new hypotheses for future testing.

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