0026-895X/04/6605-1180–1191$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2004 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 66:1180–1191, 2004

Vol. 66, No. 5
2139/1181606
Printed in U.S.A.

The Catecholamine Release-Inhibitory "Catestatin" Fragment of Chromogranin A: Naturally Occurring Human Variants with Different Potencies for Multiple Chromaffin Cell Nicotinic Cholinergic Responses

Sushil K. Mahata, Manjula Mahata, Gen Wen, William B. Wong, Nitish R. Mahapatra, Bruce A. Hamilton, and Daniel T. O'Connor

Department of Medicine and Center for Molecular Genetics, University of California; and VA San Diego Healthcare System, San Diego, California

Received April 29, 2004; accepted August 19, 2004

ABSTRACT

The catestatin fragment of chromogranin A is an endogenous inhibitor of nicotinic cholinergic transmission, functioning in negative feedback control of catecholamine secretion. We explored naturally occurring polymorphisms in the amino acid sequence of catestatin. Three human variants were identified: Gly364Ser, Pro370Leu, and Arg374Gln. Variants were tested for ability to inhibit four nicotinic processes. The rank order of potency for inhibition of catecholamine secretion was Pro370Leu > wild type > Gly364Ser > Arg374Gln. Decrease in potency was paralleled by decline in Hill slope, suggesting that negative cooperativity at ascending dose might underlie loss of potency. Several lines of evidence indicated that each variant acted as a nicotinic antagonist: potency to inhibit secretion paralleled inhibition of agonist-triggered $^{22}Na^{+}$ uptake ($r = 0.986$); variants inhibited secretion with similar potency when triggered by several nicotinic agonists, though not by agents using other secretory pathways or bypassing the nicotinic receptor; and blockade of secretion was noncompetitive with agonist. Variants also inhibited desensitization of secretion after prior agonist exposure and stimulation of secretory protein biosynthesis by agonist. Rank order of variant inhibitory potency for all four nicotinic processes was identical (Pro370Leu > wild type > Gly364Ser > Arg374Gln), suggesting mediation by similar combinations of receptor α/β subunits and that crucial catestatin residues are likely to be identical across the four processes. When catestatin variants were mixed in likely heterozygotic (1:1 M ratio) combinations, the inhibitory curve was left-shifted onto that of the more potent variant in the combination, suggesting phenotypic dominance. The results have quantitative implications for interindividual variations in human nicotinic signaling.

Chromogranin A (CGA), a 48-kDa acidic polypeptide, is the major soluble protein in the core of catecholamine storage vesicles (O'Connor et al., 1984; Winkler and Fischer-Colbrie, 1992). After nicotinic cholinergic stimulation, CGA is corereleased by exocytosis along with catecholamines from storage vesicles in adrenal medulla and sympathetic axons, and its corelease documents exocytosis as the mode of physiologic catecholamine release in experimental animals and in humans (Takiyyuddin et al., 1990). CGA is also a pro-protein giving rise to biologically active peptides such as the dysglycemic hormone pancreastatin (Tatemoto et al., 1986), the vasodilator vasostatin (Aardal et al., 1993), and catestatin (human CGA352–372), which inhibits catecholamine release in negative feedback fashion (Mahata et al., 1997). We established catestatin antagonism of nicotine-evoked catecholamine secretion in vitro in PC-12 and bovine chromaffin cells (Mahata et al., 1997, 2000), ex vivo in the perfused rat adrenal gland (Mahata et al., 2000), and in vivo in mice expressing a CGA promoter/luciferase reporter transgene (Mahata et al., 2003). If the catestatin catecholamine-release-inhibitory system operates in vivo in human, this system may present a novel target for intervention in the process of catecholamine release.

CGA is overexpressed by chromaffin cells in rodent models of both genetic (spontaneously hypertensive rat) (Schober et al., 1989; O'Connor et al., 1999) and acquired (renovascular) (Takiyyuddin et al., 1993) hypertension, and twin studies demonstrate the heritability of plasma CGA concentration in

This work was supported by grants from the Department of Veterans Affairs and the National Institutes of Health.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.002139.

ABBREVIATIONS: CGA, chromogranin A; FH+, positive family history of hypertension; SNP, single-nucleotide polymorphism; PACAP, pituitary adenylyl cyclase-activating protein; ANOVA, analysis of variance.

humans (Takiyyuddin et al., 1995). In human essential (hereditary) hypertension, the plasma concentration of catestatin is diminished not only in established cases but also in the still-normotensive offspring of patients with hypertension (FH+), (O'Connor et al., 2002), suggesting that an early deficiency of this catecholamine release inhibitory peptide might play a pathogenic role in the subsequent development of the disease.

To understand how heredity controls CGA formation and catestatin release in humans, we undertook systematic polymorphism discovery at the human *CHGA* locus (Wen et al., 2004). In the catecholamine release-inhibitory (catestatin; human CGA352–372) region, three novel amino acid substitution (nonsynonymous) single nucleotide polymorphisms were discovered (Table 1): Gly364Ser, Pro370Leu, and Arg374Gln. As many as ~4% of human chromosomes encoded one or another of these catestatin amino acid variants. Gly364Ser represents a change to an amino acid (Ser364) not previously seen at this sequence position in any mammal; Pro370Leu is a reversion of the wild-type human amino acid Pro370 to the amino acid Leu370 seen in all nonprimate mammals; and Arg374Gln disrupts the usual dibasic processing site (Arg373Arg374) flanking the carboxyl terminus of catestatin.

In this study, these naturally occurring catestatin variants were synthesized and their ability to inhibit multiple nicotinic cholinergic-stimulated processes was evaluated in chromaffin cells. We found substantial (>60-fold) differences in potency among the variants; two variants displayed loss of potency and one variant actually gained potency over the wild-type peptide. The results have quantitative implications for nicotinic cholinergic signaling in vivo.

Materials and Methods

Catestatin SNP Discovery: Resequencing the Human *CHGA* Gene. Genomic DNA from 180 persons belonging to four ethnic ancestry groups (European, sub-Saharan African, east Asian, and Mexican-American) was resequenced at the *CHGA* locus, as described previously (Wen et al., 2004). Each exon, exon/intron border, and proximal promoter was resequenced. Relatively unusual (<5% minor allele frequency) SNPs were confirmed by re-sequencing in multiple individuals and/or from the reverse direction.

Synthesis of Human Catestatin Variants. Amino acid positions are numbered according to the mature CGA protein, after excision of the amino-terminal signal peptide. Particular CGA regions, including linear, human catestatin CGA352–372 (S^{352}SMKLSFRARGYGFRGPGPQL^{372}), or its point variants (Gly364Ser, Pro370Leu, or Arg374Gln), were synthesized by the solid-phase method, using 9-fluorenylmethoxycarbonyl protection chemistry, as described previously (Mahata et al., 2000) (Tables 1 and 2). Variant Arg374Gln disrupts the usual dibasic processing site (Arg373Arg374) flanking the carboxyl terminus of catestatin. Because this processing site is normally used in catestatin biosynthesis (Taylor et al., 2000; Lee et al., 2003), we synthesized a catestatin variant (SSMKLSFRARAYGFRGPGPQL[R**Q**^{374}]GWRPSSREDSLEAGLPLQVRGYPEE) which included the 25 amino acids flanking the carboxyl terminus of catestatin, up to (but not including) the next dibasic site used in processing (Lys400Lys401). Peptides were purified to >95% homogeneity by preparative reversed-phase high-performance liquid chromatography on C-18 silica columns. Authenticity and purity of peptides were further verified by analytical chromatography (reversed-phase high-performance liquid chromatography), and electrospray-ionization or matrix-assisted laser desorption ionization mass spectrometry.

Agonist Stimulation of Catecholamine Secretion: Effect of Catestatins. Rat PC-12 pheochromocytoma cells were grown at 37°C with 6% CO_2, in six-well plates, in Dulbecco's modified Eagle's medium/high glucose medium supplemented with fetal bovine and horse serum, and penicillin/streptomycin. After cell splitting to ~50% confluence on the day before each experiment, cells were then studied at ~80% confluence. Each experimental point is derived separately from studies of a particular condition (basal versus stimulation) in three wells of a six-well plate. Norepinephrine secretion from PC-12 cells was monitored as described previously (Mahata et al., 1996). In brief, cells were labeled for 3 h with 1 μCi of [^{3}H]L-norepinephrine (71.7 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) and then incubated for 30 min with or without the secretagogue nicotine (60 μM), in the presence or absence of peptide antagonists (0.1 to 40 μM). In separate experiments, other nicotinic agonists (epibatidine, anatoxin) were tested at their optimal stimulatory concentrations (Mahata et al., 1999). Release medium and cell lysates were assayed for [^{3}H]L-norepinephrine by liquid scintillation counting. Net secretion is calculated as nicotine-stimulated release minus basal catecholamine release; in each case, mean values (± 1 S.E.M.) for three separate wells are reported, for each experimental condition.

To establish the specificity of secretory inhibition by catestatin (Mahata et al., 1997), in some experiments catecholamine release was triggered with other classes of chromaffin cell secretagogues: membrane depolarization (55 mM KCl), ATP P2x (100 μM ATP), K^+ channel blockade (2 mM $BaCl_2$, in the absence of extracellular Ca^{2+}), Ca^{2+} ionophore (1 μM ionomycin), or peptidergic (200 nM PACAP).

Agonist Desensitization of Nicotinic Cholinergic-Stimulated Catecholamine Release: Effect of Catestatins. Catestatin also inhibits desensitization of catecholamine release by prior nicotinic cholinergic stimulation (Mahata et al., 1999). Cells were preloaded with [^{3}H]L-norepinephrine and exposed to nicotinic agonists

TABLE 1

Sequence variants in human catestatin (human chromogranin $A_{352–372}$): inter-species homologies in humans and other mammals

Amino acids at positions variant in human catestatin are shown in bold type. The typical dibasic proteolytic cleavage site at the carboxy-terminal side of catestatin is given in brackets, [RR]. For human chromogranin A, this [RR] site is $Arg_{373}Arg_{374}$.

Species	Amino Acid Sequence	Variant (Frequency)	Catestatin Region in CGA
Mouse	RSMRLSFRTRGY**G**FRDPG**L**QL[R**R**]		$CGA_{364–384}$
Rat	RSMRLSFRARGY**G**FRDPG**L**QL[R**R**]		$CGA_{367–387}$
Cow	RSMRLSFRARGY**G**FRGPG**L**QL[R**R**]		$CGA_{344–364}$
Pig	RSMRLSFRAPAY**G**FRGPG**L**QL[R**R**]		$CGA_{343–363}$
Horse	RSMKLSFRARAY**G**FRGPG**L**QL[R**R**]		$CGA_{343–363}$
Chimp	SSMKLSFRARAY**G**FRGPG**P**QL[R**R**]		$CGA_{354–374}$
Human			
Wild-type	SSMKLSFRARAY**G**FRGPG**P**QL[R**R**]		$CGA_{352–372}$
Variant	SSMKLSFRARAY**S**FRGPG**P**QL[R**R**]	Gly364Ser(3.1%)	
Variant	SSMKLSFRARAY**G**FRGPG**L**QL[R**R**]	Pro370Leu(0.6%)	
Variant	SSMKLSFRARAY**G**FRGPG**P**QL[R**Q**]	Arg374Gln(0.3%)	

(30 μM) for periods of 10 min (incubation I). Cells were then washed twice (5 min each) in secretion buffer as described above and rechallenged with nicotinic agonists (30 μM) for a period of 10 min (incubation II), after which the cells were harvested for measurement of norepinephrine release from cells and medium.

Nicotinic Cholinergic Stimulated Transcription of CGA: Effect of Catestatins. Nicotinic stimulation also triggers the biosynthesis of CGA at the transcriptional level, representing an example of stimulus-transcription (stimulus-secretion-synthesis) coupling (Tang et al., 1996).

Plasmid pHJLD5, in which a functional 1133-bp mouse CGA promoter drives expression of a luciferase reporter, has been described previously (Wu et al., 1991). The mouse CGA promoter region in this plasmid extends from −1133 bp upstream of the transcription initiation (cap) site, to +42 bp downstream of the cap site. This transfected promoter responds to nicotinic cholinergic stimulation (Tang et al., 1996). Supercoiled plasmids were prepared on Qiagen columns (QIAGEN Inc., Valencia, CA).

One day before transfection, PC-12 cells were split onto 6-cm plastic plates coated with poly-D-lysine (Sigma, St. Louis, MO) at 40 to 50% confluence and transfected with 1 μg of supercoiled luciferase reporter plasmid DNA per plate, using the efficient liposome (Mahapatra et al., 2003) method (Superfect; QIAGEN)

After transfection, nicotinic cholinergic effects on the transfected CGA promoter were established by adding nicotine (1 mM; Sigma) (or vehicle) to PC-12 cells; 1 mM nicotine is the dose eliciting maximal CGA transcription in this system, and the effect is receptor-specific (Tang et al., 1996). 18 to 20 h after transfection, cell were harvested and lysed (100 μl/plate) for assay of luciferase (light units) and protein (Tang et al., 1996).

Nicotinic Cholinergic Signal Transduction: $^{22}Na^+$ Uptake by Chromaffin Cells. $^{22}Na^+$ uptake was performed as described previously (Mahata et al., 1997) with minor modifications. Before experiments, PC-12 cells were washed twice with 50 mM Na^+/sucrose medium containing 50 mM NaCl, 187 mM sucrose, 5 mM KCl, 2 mM $CaCl_2$, and 5 mM HEPES adjusted to pH 7.4 with NaOH. To measure $^{22}Na^+$ influx, this medium was then supplemented with 5 mM ouabain (to prevent active extrusion of newly taken up $^{22}Na^+$ from cells) and 1 μCi/ml $^{22}NaCl$ (PerkinElmer Life and Analytical Sciences; specific activity, 868.37 mCi/mg). Incubation was carried out at 22°C for 5 min in the presence or absence of secretagogues, and then the cells were washed within 10 s with three changes (1 ml each) of 50 mM Na^+/sucrose medium with 5 mM ouabain. The cells were lysed (see above), and $^{22}Na^+$ taken up into the cell lysate was measured in a γ counter (LKB 1274 RIAGAMMA; Amersham Biosciences, Piscataway, NJ). The data were expressed as dpm/well, minus background (uptake in the absence of agonist).

Statistical Analysis. Experiments with catestatin variants were repeated at least three times, with three wells per condition in each experiment. Curve fitting was accomplished in the program Kaleidagraph (Synergy Software, Reading, PA), using the Stineman function, which applies a geometric weight ± 10% of the data range, to arrive at a smoothed curve. The IC_{50} value of a catestatin variant is interpolated as the concentration that achieved 50% inhibition of nicotinic-stimulated catecholamine release or desensitization. Slopes, intercepts, IC_{50} values, and error terms were computed in Prism 2.0 (GraphPad Software, San Diego, CA). Results are expressed as the mean value ± S.E.M. When only two conditions (e.g., control and experimental) are compared, the data are evaluated by unpaired t tests. When multiple conditions (e.g., several catestatin variants) are compared, one-way ANOVA is used, followed by Dunnett's multiple comparison post hoc tests, if appropriate. Descriptive and inferential statistics are performed with the program InStat (GraphPad Software). Significance is determined at the $P \leq 0.05$ level.

Results

Identification of Nonsynonymous Single Nucleotide Polymorphisms in the Catestatin Sequence. To identify human genetic variants in catestatin that might alter its function, we resequenced the gene from 180 ethnically diverse subjects (Wen et al., 2004), as well as a nonhuman primate species (chimpanzee). Three nonsynonymous (amino acid replacement) polymorphisms (Gly364Ser, Pro370Leu, and Arg374Gln; codon positions in the mature CGA protein; minor allele frequencies, 0.3–3.1%) were discovered as human variants in the catestatin peptide region (human $CGA^{352–372}$) (Table 1).

Alignment of catestatin sequences at human codon 364 (Table 1) revealed that Gly364 is conserved among seven mammalian species for which sequence is available, but heterozygous substitution to Ser364 occurred in 11 of 180 human subjects. The Gly364Ser variant is distributed across three ethnic groups (5 of 88 Asians, 5 of 102 white persons, 1 of 56 Hispanics), but absent from the African-American samples (0 of 114, $p < 0.01$ by χ^2).

Although Leu370 is the normal allele in all reported nonprimate mammals, codon 370 specifies Pro370 in primates (human and chimpanzee). Reversion of Pro370 → Leu370 (in Pro370Leu) occurred in 2 of 180 subjects, both African-American.

The dibasic site Arg373Arg374 is conserved across all species, including primates (Table 1). This dibasic site is used in the formation of catestatin (Taylor et al., 2000; Herrero et al., 2002; Lee et al., 2003). In 1 of 180 human subjects (i.e., 1 of 360 chromosomes), Arg374 becomes Gln374. Because replacement of Arg374 by Gln374 disrupts the usual dibasic processing site (Arg373Arg374) at the carboxyl-terminal side of catestatin ($CGA^{352–372}$), the Arg374Gln variant is likely to disrupt proteolytic excision of active catestatin from CGA, resulting in generation of a longer catestatin peptide (Taylor et al., 2000; Herrero et al., 2002; Lee et al., 2003), perhaps extending to the next dibasic site typically used, Lys400Lys401 (Herrero et al., 2002) (Table 2).

Relative Potencies of Human Catestatin Variants on Nicotinic Cholinergic-Evoked Catecholamine Secretion. All four human catestatins (wild-type and three variants) inhibited 60 μM nicotine-stimulated catecholamine re-

TABLE 2

Human catestatin variant synthetic peptides

Variant amino acid residues are given in bold type. The position of the dibasic processing site at wild-type residues [$Arg_{373}Arg_{374}$] is given in brackets in the variant Arg374Gln, as [RQ_{374}]. Peptide Arg374Gln bears a carboxy-terminal extension up to the residue (Glu_{399}) adjacent to the next dibasic processing site ($Lys_{401}Lys_{402}$).

Human catestatin variant	Primary Structure of the Synthetic Variant Peptide
Wild-Type ($CGA_{352–372}$)	SSMKLSFRARAYGFRGPGPQL
Gly364Ser	SSMKLSFRARAYS$_{364}$FRGPGPQL
Pro370Leu	SSMKLSFRARAYGFRGPGL$_{370}$QL
Arg374Gln ($CGA_{352–399}$)	SSMKLSFRARAYGFRGPGPQL[RQ_{374}]GWRPSSREDSLEAGLPLQVRGYPEE

lease from chromaffin cells, although their potencies (IC_{50} values) varied widely (Fig. 1A). Compared with wild type (IC_{50} 0.82 ± 0.02 μM), Pro370Leu actually had increased potency (IC_{50} 0.37 ± 0.03 μM), whereas Gly364Ser displayed moderate (IC_{50} 3.65 ± 0.11 μM) and Arg374Gln severe (IC_{50} 22.5 ± 4.05 μM) decreases in potency. The potency rank

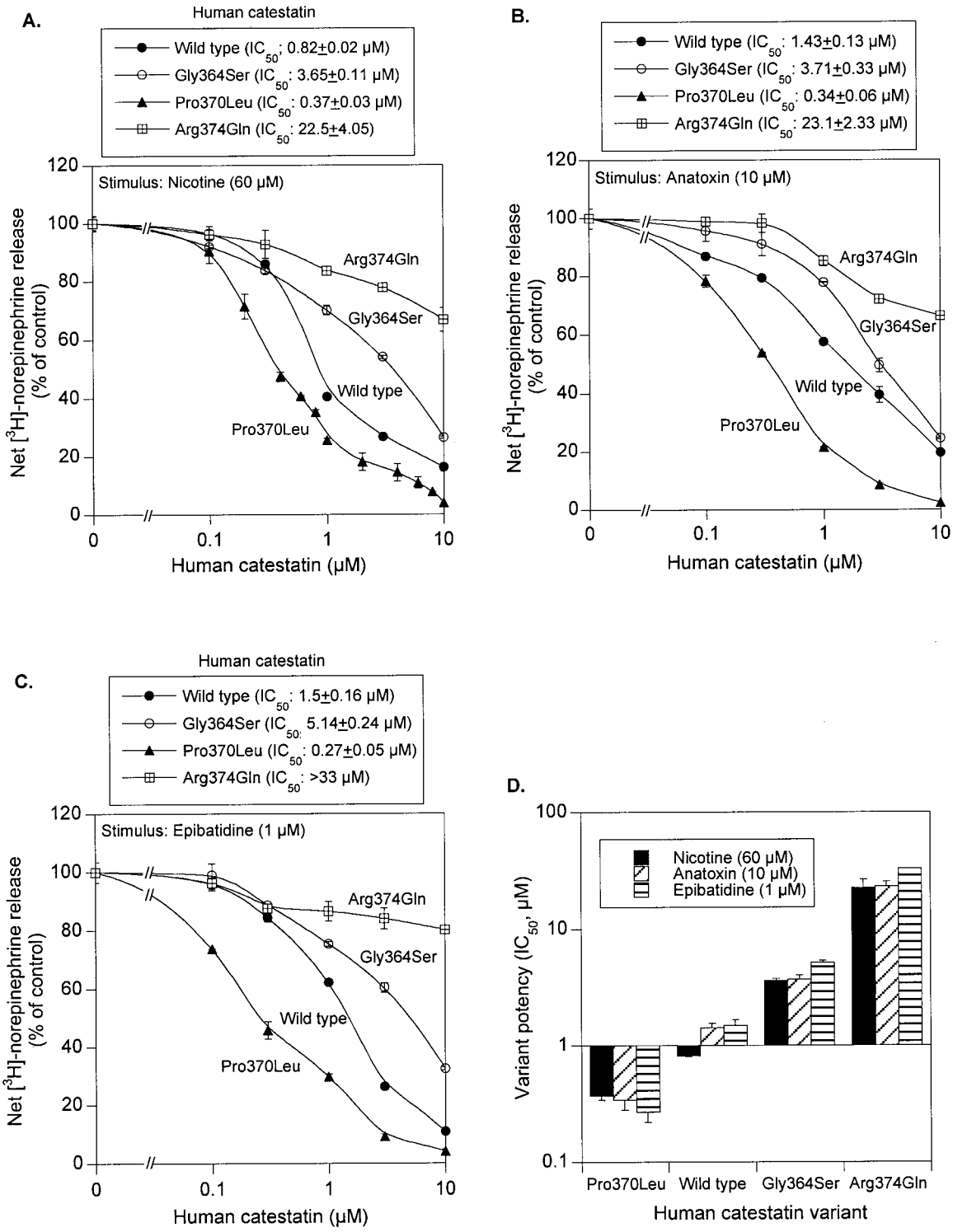

Fig. 1. Potency of catecholamine secretion inhibition by human catestatin variants, when secretion is triggered by nicotinic cholinergic agonists. A, peptide inhibitory potency during secretory stimulation by nicotine (60 μM). B, peptide inhibitory potency during secretory stimulation by anatoxin (10 μM). C, peptide inhibitory potency during secretory stimulation by epibatidine (1 μM). PC-12 cells prelabeled with [^{3}H]L-norepinephrine were incubated with 60 μM nicotine (or other agonist), either alone or in combination with logarithmically ascending doses of each catestatin variant (0.1–10 μM) for 30 min. Each data point (including control) represents mean data from three separate wells. Control (100%) net norepinephrine release is that in the presence of nicotine (60 μM) stimulation alone, without catestatin or its variants. D, correlation of peptide inhibitory potencies during secretory stimulation by the nicotinic cholinergic agonists nicotine (60 μM), with stimulation by anatoxin (10 μM; r = 0.999), or epibatidine (1 μM; r = 0.999). The peptide IC_{50} rank order was also parallel for anatoxin and epibatidine (r = 0.999).

order was thus: Pro370Leu > wild type > Gly364Ser > Arg374Gln.

In addition to nicotine, the variants were tested for ability to inhibit the effects of two other nicotinic cholinergic agonists on catecholamine secretion: anatoxin (10 μM; Fig. 1B) and epibatidine (1 μM; Fig. 1C). The rank order of peptide inhibitory potencies was identical for the three nicotinic agonists (nicotine, anatoxin, epibatidine; Fig. 1D), and the peptides' IC_{50} values for nicotine predicted their IC_{50} values for both anatoxin ($r = 0.999$) and epibatidine ($r = 0.999$). The four peptides' IC_{50} values did not differ among the three agonists (ANOVA $F = 0.077$, $p = 0.926$).

Because these different nicotinic agonists (Badio and Daly, 1994; Mahata et al., 1999, 2002) might also differ in potency and efficacy, we present data on these parameters for each agonist (nicotine, anatoxin, epibatidine) in our experimental system, PC-12 chromaffin cells (Table 3). Although the potencies of these three agonists were quite different (from 0.015 to 8.4 μM), their maximal effects on catecholamine secretion were generally similar (from 24.4 to 40.4%). In these secretion experiments on catestatin effects (Fig. 1), we used agonist concentrations ~5 to 10-fold higher than the EC_{50} values (Table 3) to achieve substantial baseline values for catestatin inhibition.

Efficacies (Maximal or Ceiling Effects) of Catestatin Variants on Nicotine-Induced Catecholamine Release. Because the catestatin variants displayed such a wide (~60-fold) range of inhibitory potencies (IC_{50}, 0.37–22.5 μM), we probed the ceiling effect of these variants. At very high doses (40 μM; Fig. 2), three of the catestatins (Pro370Leu, wild-type, and Gly364Ser) showed virtually complete (~100%) inhibition of nicotine-triggered catecholamine secretion. The least potent version (Arg374Gln; IC_{50}, 22.5 μM) achieved only ~60% inhibition at 40 μM, but greater doses of Arg374Gln were not practical in this system.

Specificity of Human Catestatin Variants: Effects on Different Mechanisms of Secretagogue-Induced Catecholamine Secretion. In addition to the nicotinic-cholinergic stimulation, we tested secretagogues that act at stages in the secretory pathway later than the nicotinic receptor (Mahata et al., 1997), including membrane depolarization (by K^+) to open voltage-gated calcium channels, an alkaline earth (Ba^{2+}) to block cell surface K^+ channels and thereby depolarize the cell membrane, or a Ca^{2+} ionophore (ionomycin) to admit extracellular Ca^{2+} to the cytosol. In addition, we used secretagogues that function on different targets from the nicotinic-cholinergic receptor, such as ATP acting on the P2x purinergic receptor or PACAP acting on the PAC1 G-protein-coupled receptor (Taupenot et al., 1998, 1999). Catestatin and its variants suppressed norepinephrine release only when triggered by nicotine (Fig. 3), but not when secretion was caused by agents acting later (i.e., distal to the receptor) in the nicotinic secretory pathway, or acting on other receptor classes.

Effect of Human Catestatin Variants on Initial Nicotinic Cationic Signal Transduction. Because the initial step in nicotinic cholinergic signal transduction is receptor-mediated entry of extracellular Na^+ into the cell, we predicted that each variant should inhibit this process. Nicotine resulted in translocation of $^{22}Na^+$ into chromaffin cells, and the process was inhibited by all four of the catestatin variants (Fig. 4A), establishing each as an antagonist at the nicotinic receptor. The variants' rank order of inhibition of $^{22}Na^+$ uptake paralleled their potencies for inhibition of catecholamine secretion (Fig. 4B; $r = 0.986$): Pro370Leu > wild type > Gly364Ser > Arg374Gln.

Nicotinic Antagonism by Catestatin Variants: Competitive Versus Noncompetitive with Agonist. To probe the receptor site of the variants' nicotinic inhibition, we tested whether very high doses of nicotinic agonist could overcome the inhibition (Fig. 5). We treated PC-12 cells with log_{10}-ascending doses of nicotine (10 to 1000 μM) either alone or with ascending doses of catestatin variants (0.1 to 10 μM) for 30 min, after which cells were harvested for measurement of norepinephrine release. Even very high doses of nicotine (1000 μM) could not overcome the catestatin variants' inhibition of norepinephrine release at high catestatin dose (10 μM) (Fig. 5), thereby functionally establishing the variants as noncompetitive nicotinic antagonists, at least under the conditions tested here.

TABLE 3

Differential potencies (EC_{50} values) and efficacies (maximal or "ceiling" responses) of three nicotinic agonists to stimulate catecholamine secretion from PC-12 chromaffin cells

Results are shown as the mean value for three replicate experiments.

Agonist	Maximal secretion response		EC_{50}
	Dose	Percentage (± S.E.M.)	
	μM		μM
Nicotine	100	24.4 ± 0.64	8.4
Epibatidine	0.1	38.6±0.2	0.015
Anatoxin	10	40.4±0.9	1.4

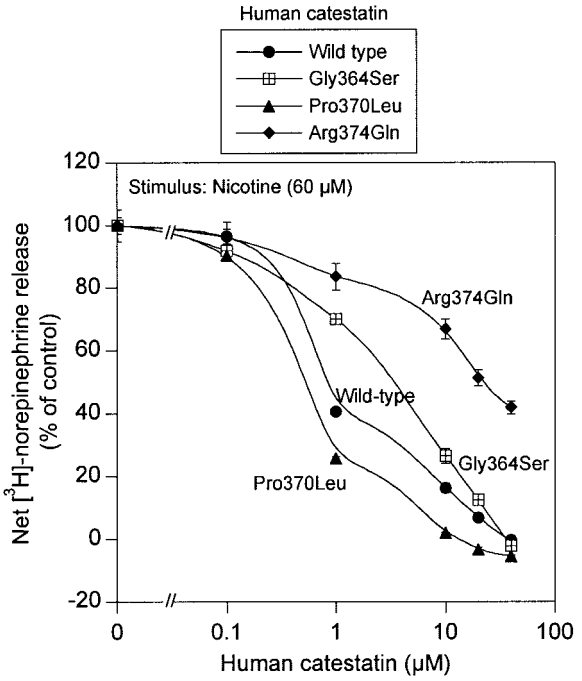

Fig. 2. Efficacy (maximal or ceiling effect, at high dose) of catestatin variants to inhibit catecholamine secretion. PC-12 cells were prelabeled with [^{3}H]L-norepinephrine, and then incubated with 60 μM nicotine, either alone or in combination with logarithmically ascending doses of each catestatin variant (0.1 to 40 μM) for 30 min. Control (100%) net norepinephrine release is that in the presence of nicotine (60 μM) stimulation alone, without catestatin variants. Each data point (including control) represents mean data from three separate wells.

Testing the Phenotypically Dominant/Recessive Nature of the Human Catestatin Variants. Noting the very wide spectrum of inhibitory potencies (IC_{50}, ~0.37–22.5 μM), we wondered about the consequences of coexpression of the wild-type catestatin with a variant in heterozygotes; in particular, we wondered if one variant is phenotypically dominant over another in its actions on catecholamine release. Because the variants are relatively uncommon (minor allele frequencies 0.3–3.1%; Table 1), compound variant heterozygotes are unlikely to occur frequently; thus, we tested each variant in combination with the wild-type version. When two different catestatins (wild-type plus variant) were tested together, a 50:50 M mixture was used, to achieve the same final concentration as pure, unmixed control (wild-type) peptide.

In each case, the more potent catestatin displayed phenotypic dominance over the less potent version (i.e., the mixture curve was left-shifted onto the more potent curve): wild-type dominant over Gly364Ser (Fig. 6A) or Arg374Gln (Fig. 6B); and Pro370Leu (Fig. 6C) dominant over wild type.

Role of Cooperativity in the Catestatin Variants' Inhibition of Secretion. Hill plots assessed the fractional effect of catestatin to inhibit nicotinic-stimulated catecholamine secretion as a function of peptide dose (Fig. 7). For each catestatin variant, the plots were linear over a wide range of catestatin concentrations (2 orders of magnitude). Hill slopes were near unity for the wild-type (slope = 1.06 ± 0.163; Fig. 7A) and more potent variant (Pro370Leu, slope = 0.997 ± 0.072; Fig. 7C) but fell for the less potent versions (Gly364Ser, slope = 0.73 ± 0.036, Fig. 7B; Arg374Gln, slope = 0.55 ± 0.044, Fig. 7D). Indeed, fall in potency paralleled fall in Hill slope (r = 0.989; Fig. 7E), suggesting that relatively negative cooperativity at ascending doses might underlie, at least in part, the loss of potency in particular variants, especially for Arg374Gln.

Effect of Catestatin Variants on Desensitization of Catecholamine Release. Nicotinic cholinergic processes are subject to desensitization after repeated exposure to agonist, and catestatin can block this phenomenon (Mahata et al.,

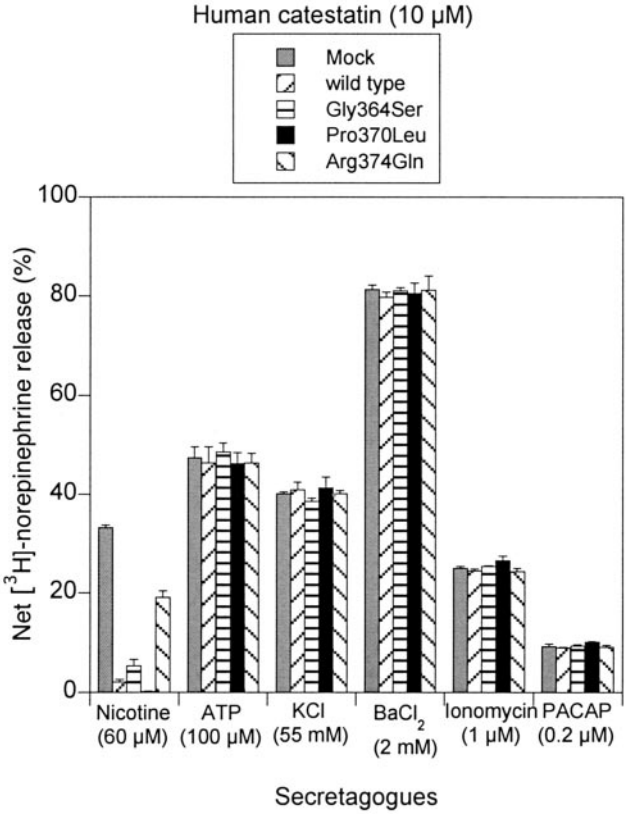

Fig. 3. Catestatin variants' inhibitory effect on catecholamine secretion is specific for nicotinic cholinergic stimulation. PC-12 cell vesicular catecholamine stores were prelabeled with [^{3}H]L-norepinephrine and were then treated with one of several secretagogue classes [nicotinic cholinergic stimulation by 60 μM nicotine; membrane depolarization by 55 mM extracellular K^+; P2x receptor activation by 100 μM ATP; K^+ channel blockade by 2 mM Ba^{2+}; calcium ionophore 1 μM ionomycin; or peptidergic (PAC1) stimulation by 0.2 μM PACAP], either alone or in combination with catestatin variants (10 μM), and harvested after 30 min for measurement of norepinephrine secretion. Each data point (including control) represents mean data from three separate wells.

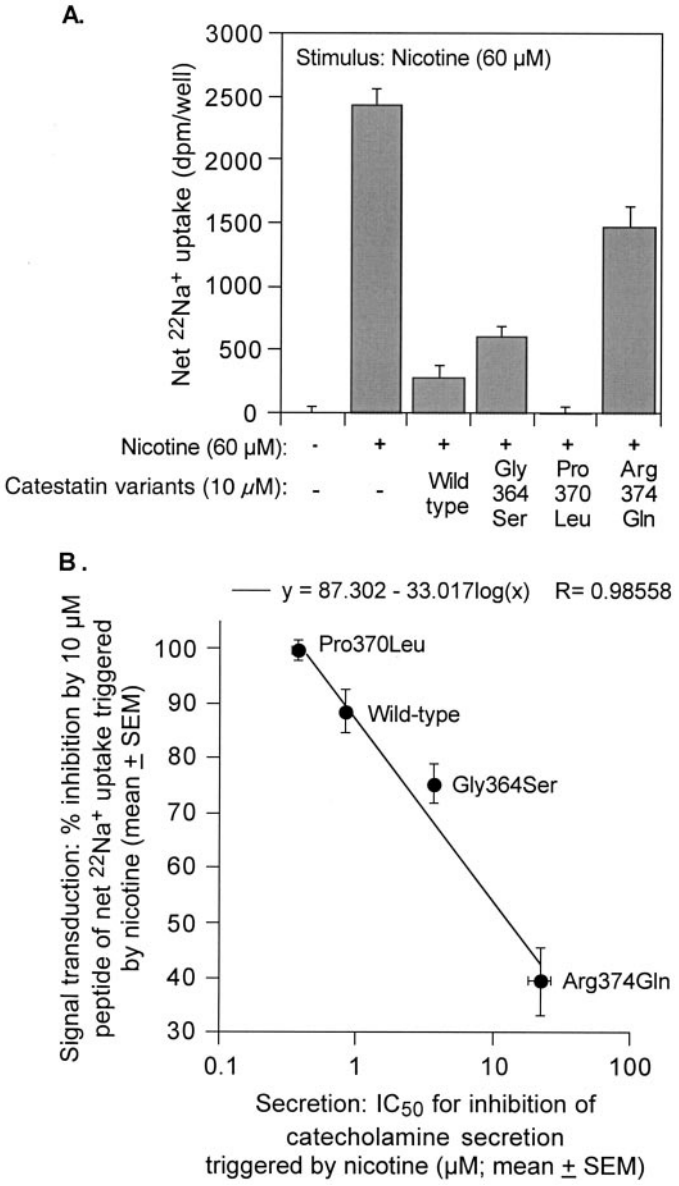

Fig. 4. Effect of catestatin variants on initial nicotinic cholinergic signal transduction (agonist-triggered entry of extracellular Na^+ into the cell). The effect of the variants was tested on 60 μM nicotine-stimulated uptake of $^{22}Na^+$ by PC-12 cells over 5 min. Results are plotted as inhibition of $^{22}Na^+$ uptake by 10 μM peptide versus secretory inhibitory potency (IC_{50}) for the same peptide (A). Catestatin variant effects on the two processes paralleled each other (r = 0.986) (B). Each data point (including control) represents mean data from three separate wells.

1999). In this study, we found that each catestatin variant blocked agonist-induced desensitization of catecholamine secretion in dose-dependent fashion (Fig. 8), in rank order of potency (IC_{50}): Pro370Leu > wild type > Gly364Ser > Arg374Gln.

Catestatin Variants and Secretory Protein Transcription. Nicotinic cholinergic agonists trigger not only release of catecholamines, CGA, and catestatin, but also resynthesis of the just-released transmitters, by the process of stimulus-secretion-synthesis (i.e., stimulus-transcription) coupling (Tang et al., 1996). Using nicotine, we triggered transcription of a CGA promoter/luciferase reporter transfected into chromaffin cells (Fig. 9), achieving 2.9-fold activation over basal transcription. The variants each dose dependently inhibited the transcriptional activation (Fig. 9), with IC_{50} values in this rank order: Pro370Leu > wild type > Gly364Ser > Arg374Gln.

Catestatin Variants: Parallel Potencies to Inhibit Several Nicotinic Agonist-Triggered Processes: Catecholamine Secretion, Desensitization of Secretion, and Secretory Protein Transcription. The catestatin variants' inhibitory potencies displayed similar rank orders (Fig. 10) for the three nicotinic processes affected. Secretion potency paralleled desensitization potency ($r = 0.997$), as well as transcription potency ($r = 0.999$). Desensitization potency also paralleled transcription potency ($r = 0.999$). The IC_{50} values of the four peptides for the three processes (secretion, desensitization, transcription) did not differ (ANOVA, $F = 0.071$, $p = 0.932$).

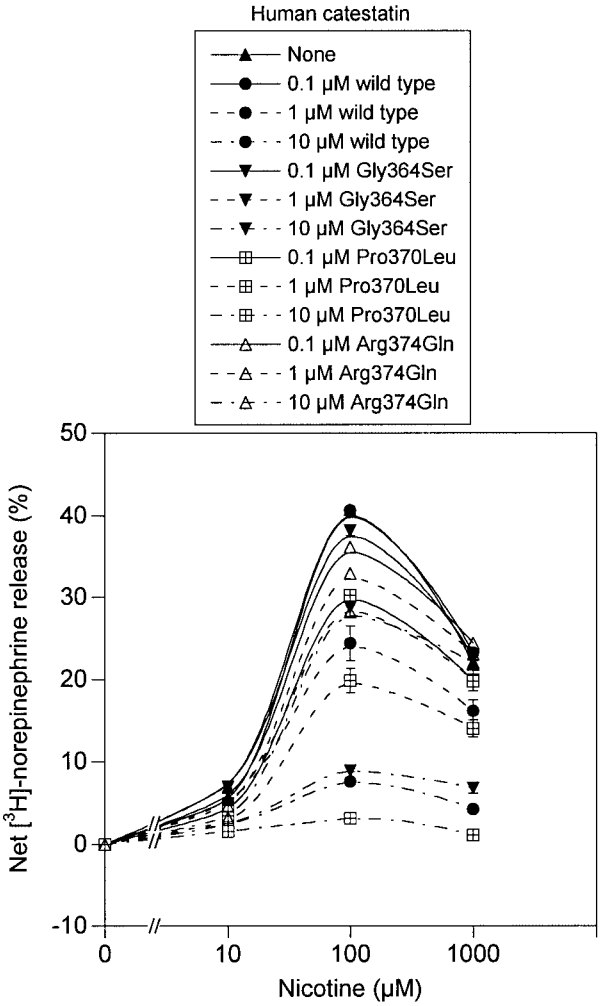

Fig. 5. Mechanism of nicotinic cholinergic secretory inhibition by the catestatin variants: competitive versus noncompetitive with agonist. To test the receptor site of nicotinic cholinergic inhibition by the peptide variants, [^{3}H]norepinephrine-loaded PC-12 cells were treated for 30 min with increasing doses of agonist (nicotine, 10–1000 μM), in the presence or absence of increasing doses of each catestatin variant (0.1–10 μM). After 30 min, cells were harvested to quantitate norepinephrine release. Each data point (including control) represents mean data from three separate wells.

Discussion

In this study, we found that three naturally occurring human variants of the catecholamine release-inhibitory (catestatin) fragment of CGA (CGA352–372) differ markedly in functionality; some variants (Ser364Gly, Arg374Gln) suffer a loss in potency, although one variant (Pro370Leu) actually gained potency to inhibit several nicotinic cholinergic responses in chromaffin cells (Figs. 1, 8, and 9).

Based on previous studies in vitro with chromaffin cells (Mahata et al., 1997, 1999, 2000), ex vivo with the superfused rat adrenal gland (Mahata et al., 2000) and in vivo with mice (Mahata et al., 2003), we previously concluded that the CGA fragment catestatin acts as a potent and specific nicotinic-cholinergic antagonist. In human essential (hereditary) hypertension, the plasma concentration of catestatin is diminished not only in established cases, but also in the still-normotensive offspring of patients with hypertension (FH^+) (O'Connor et al., 2002), suggesting that an early deficiency of this catecholamine release inhibitory peptide might play a pathogenic role in the subsequent development of the disease. Plasma catestatin concentration is bimodally distributed, with a lower mode occupied entirely by FH^+ subjects, suggesting a major gene effect on this biochemical "intermediate phenotype" for hypertension (O'Connor et al., 2002). This viewpoint is strengthened by two consonant observations in the same subjects: biochemically, the FH^+ already had increased adrenal epinephrine production and renal epinephrine excretion, and physiologically, subjects with lower plasma catestatin had greater pressor responses to environmental stimuli. Hence, catestatin concentration predicts (and may control) both biochemical and physiological consequences of efferent sympathoadrenal outflow (O'Connor et al., 2002).

We used exogenous [^{3}H]norepinephrine to label PC-12 cells' chromaffin granule catecholamine stores, as extensively described previously (Takiyyuddin et al., 1990; Mahata et al., 1996, 1997). PC-12 endogenous catecholamine vesicular stores include, in rank order: dopamine > norepinephrine > epinephrine (Greene and Tischler, 1976; Li et al., 2003; Tischler et al., 2004). In the adrenal medulla, separate chromaffin cells store and release epinephrine and norepinephrine (Bartlett and Smith, 1974); however, PC-12 cells are a homogeneous, clonal cell population (Greene and Tischler, 1976; Tischler et al., 2004). Alterations in endogenous secretion of both norepinephrine and epinephrine have been implicated in the pathogenesis of such disease states as human essential hypertension (Rumantir et al., 2000; DeQuattro and Feng, 2002), and catestatin inhibits catecholamine release not only from PC-12 cells but also from chromaffin cells in primary culture (Mahata et al., 1997), in vivo/in situ rodent chromaffin cells (Mahata et al., 2000), noradrenergic neurites (Mahata et al., 1997), and perhaps even human

chromaffin cells in vivo (O'Connor et al., 2002). Thus, the differential catestatin variant effects we have observed here (Fig. 10) are likely to have functional consequences for both epinephrine and norepinephrine release in hypertension.

Nicotinic Cholinergic Specificity of Catestatin Variant Action. Consistent with our previous results on bovine catestatin (Mahata et al., 1997, 1999, 2000) we found that human catestatin variants displayed specific antagonism for the nicotinic cholinergic receptor, without inhibition of agents that act either on different receptors or bypass the nicotinic-cholinergic receptor (Fig. 3). Blockade of nicotinic agonist-triggered entry of $^{22}Na^{+}$ into the cytoplasm (Fig. 4), coupled with the close parallel ($r = 0.986$) between variant potencies to inhibit agonist-activated $^{22}Na^{+}$ entry and catecholamine secretion, further established the site of action of the catestatin variants directly on the nicotinic receptor.

The inability of nicotinic agonists to overcome the catestatin variants' secretory inhibition, even at very high agonist doses, is consistent with noncompetitive nicotinic inhibition (Fig. 5), though we do not yet know the precise site at which catestatin interacts with the nicotinic receptor.

Hill plots, showing the fractional effect of catestatin to inhibit nicotinic-stimulated catecholamine secretion (Fig. 7), seemed to be linear over a wide range of catestatin concentrations (~2 orders of magnitude), and Hill slopes near unity for wild-type catestatin (slope = 1.06 ± 0.163; Fig. 7A) or Pro370Leu (slope = 0.997 ± 0.072; Fig. 7C) indicates noncooperativity during nicotinic inhibition. Decline in Hill slope for the less potent variants (Fig. 7, B and D) suggests that, at ascending peptide doses (especially for Arg374Gln, slope = 0.55 ± 0.044; Fig. 7D), such catestatins might actually exhibit negative cooperativity. The structural basis for this phenomenon is not clear, although catestatins do exhibit amphiphilic β sheet character in solution, with alternating cationic and hydrophobic amino acid residue side chains (Tsigelny et al., 1998; Preece et al., 2004), wherein the hydrophobic domains may contribute to formation of a hairpin (β-strand/loop/β-strand) structure by intramolecular (strand-to-strand) hydrophobic collapse; intermolecular hydrophobic interactions might result in self-association and hence diminution in the effective local concentration of the monomeric peptide.

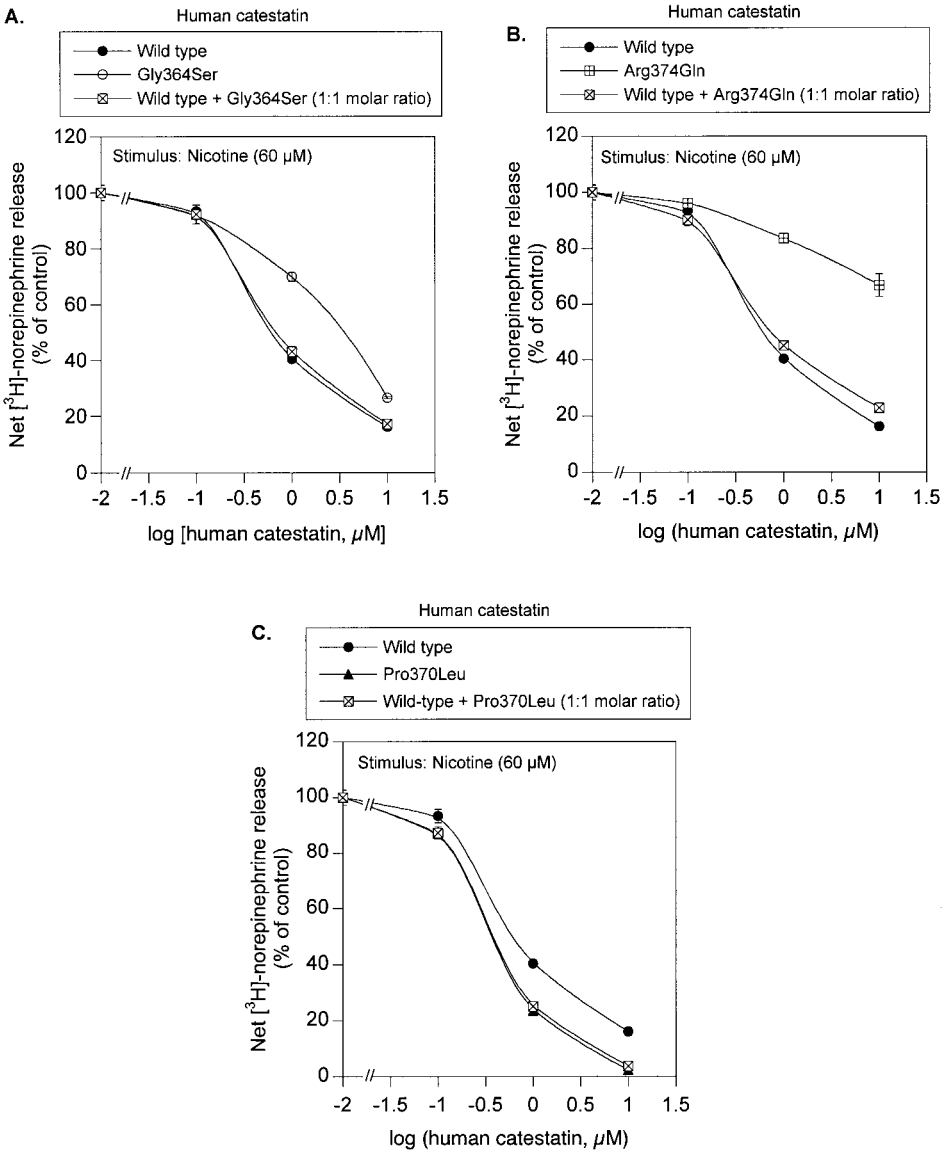

Fig. 6. Phenotypic dominant/recessive actions of catestatin variants on catecholamine secretion. PC-12 cells were treated with nicotine (60 μM), in the presence or absence of ascending doses (0.1–10 μM) of wild-type catestatin, variant catestatin (Pro370Leu, Gly364Ser, or Arg374Gln), or the two (wild-type plus variant) in 1:1 M ratio. During mixing of wild-type and variant, the total molar concentration was kept the same as for the isolated catestatins (0.1, 1, or 10 μM).

Multiple Nicotinic Cholinergic Responses Inhibited by the Catestatin Variants. Desensitization (also called tolerance, refractoriness, subsensitivity, or down-regulation) refers to loss of cell or tissue responses after repeated or prolonged application of a stimulus (Mahata et al., 1999). Consistent with previous findings (Mahata et al., 1999), we noted that catestatin variants not only blocked nicotine-evoked catecholamine release but also dose-dependently antagonized the typical effect of

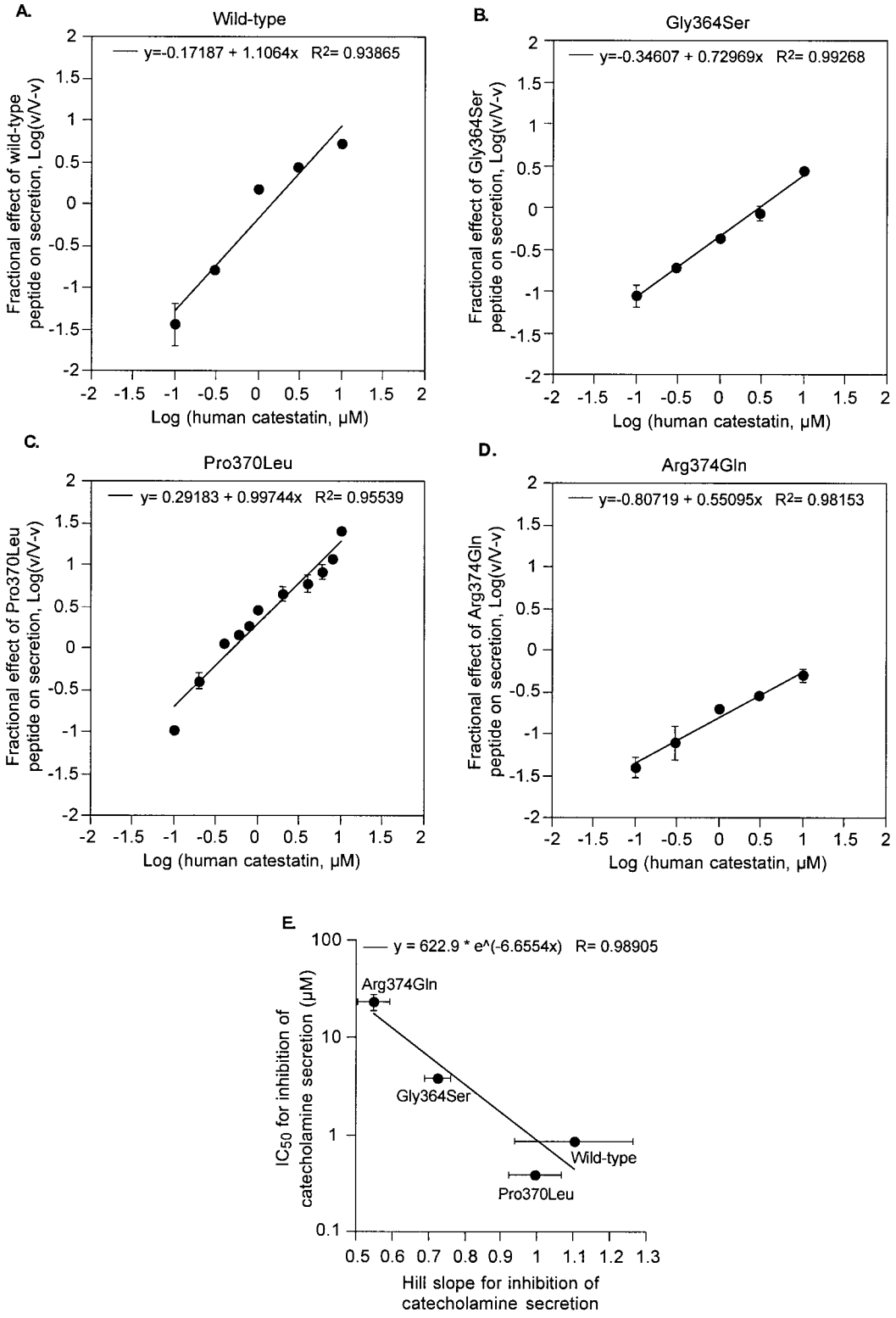

Fig. 7. Role of cooperativity in the catestatin variants' inhibition of secretion: Hill plots. The plots assess the fractional effect of ascending doses (0.1–1 μM) of catestatin variants to inhibit catecholamine release triggered by 60 μM nicotine from PC-12 cells (A–D). The slope is the Hill coefficient. The final panel plots the secretion inhibitory potency of each variant versus its Hill slope (r = 0.989) (E). Each data point (including control) represents mean data from three separate wells.

prior nicotinic agonist exposure to desensitize the effect of a subsequent nicotinic challenge to induce secretion (Fig. 8); indeed, the rank order of potencies to block these two nicotinic processes (secretion and desensitization) was identical (Pro370Leu > wild-type > Gly364Ser > Arg374Gln; Fig. 10), and the IC_{50} values correlated well ($r = 0.997$).

Likewise, the variants also blocked nicotinic stimulation of transcription of CGA in chromaffin cells (Fig. 9), thus modulating, in negative feedback fashion, the physiological process of stimulation-secretion-synthesis (i.e., stimulus-transcription) coupling (Tang et al., 1996, 1997, 1998). Once again, the rank order of potencies to block two nicotinic processes (secretion and transcription) was identical (Pro370Leu > wild-type > Gly364Ser > Arg374Gln; Fig. 10), and the IC_{50} values correlated well ($r = 0.999$). Because CGA is the precursor of catestatin (Mahata et al., 1997), nicotinic cholinergic control of both secretion and transcription in chromaffin cells seems to be regulated in autocrine, negative feedback fashion by catestatin. (Fig. 11)

Finally, variant potencies to inhibit all three nicotinic processes (secretion, desensitization, and transcription) displayed the same rank order (Pro370Leu > wild-type > Gly364Ser > Arg374Gln; Figs. 4 and 10). The variant potencies did not systematically differ among the nicotinic processes of secretion, desensitization, and transcription

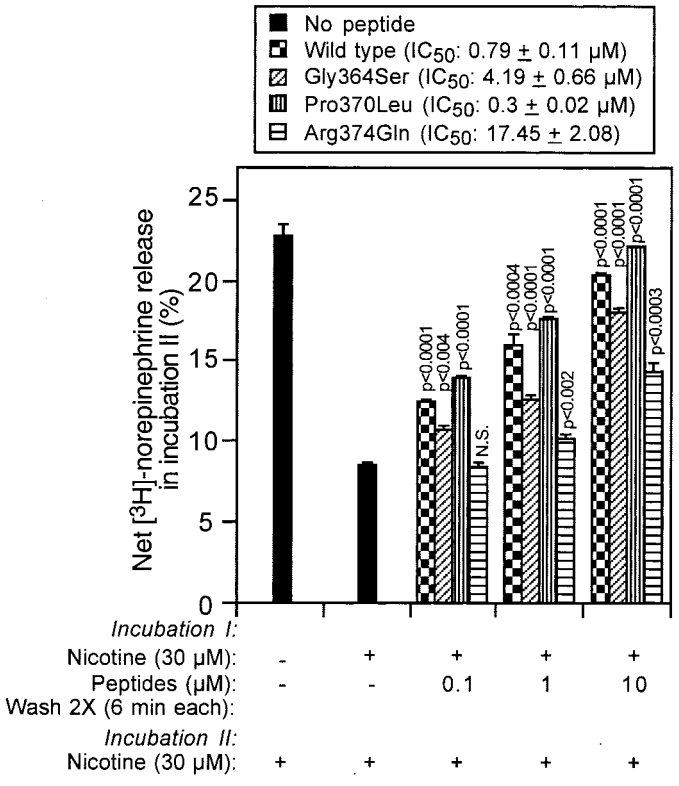

Fig. 8. Desensitization of nicotinic cholinergic triggered catecholamine release: effect of catestatin variants. PC-12 cells were pretreated (Incubation I) with an initial dose of nicotine (30 μM for 10 min), in the presence or absence of ascending doses (0.1–10 μM) of catestatin variants. Cells were washed twice (5 min each) with buffer and then exposed to a second secretory challenge (incubation II) with nicotine (30 μM for 10 min), this time in the absence of catestatins, after which medium and cells were harvested for measurement of [^{3}H]norepinephrine release. Each data point (including control) represents mean data from three separate wells. *P* values compare each peptide point to the desensitized value, by nicotine, in the absence of peptide.

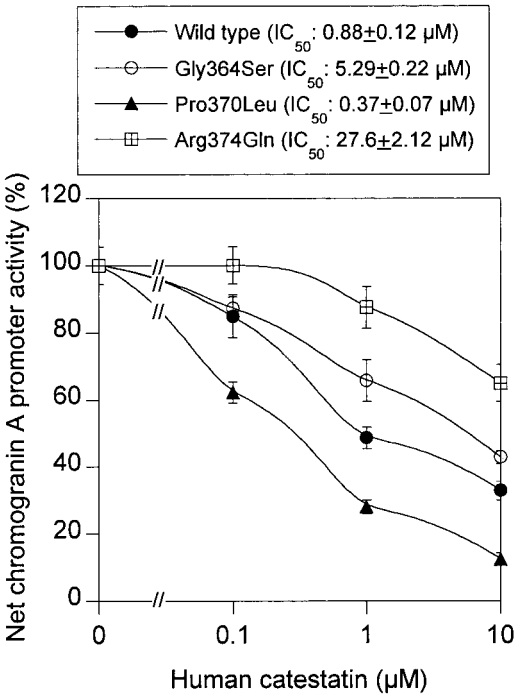

Fig. 9. Catestatin variants and secretory protein transcription after nicotinic cholinergic stimulation. PC-12 cells were transfected with a chromogranin A promoter/luciferase reporter plasmid (mouse chromogranin A 1133-bp proximal promoter). After 4 h, cells were exposed to 1 mM nicotine for an additional 18 h, in the presence or absence of ascending doses (0.1–10 μM) of catestatin variants. At the conclusion of this exposure, cells were harvested for measurement of luciferase reporter activity. Results are expressed as percentage of luciferase activity stimulated by nicotine alone, minus blank (no nicotine). In this experiment, the agonist signal/noise (nicotine/mock) ratio was 2.9:1. Each data point (including control) represents mean data from three separate wells.

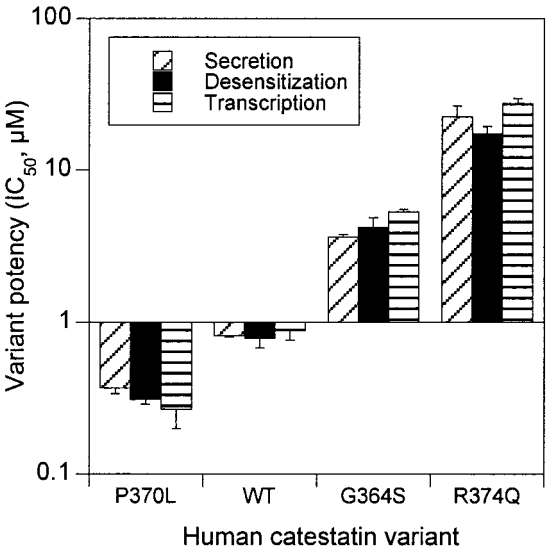

Fig. 10. Parallel inhibition of nicotinic cholinergic-triggered secretion, desensitization, and transcription by catestatin variants. IC_{50} values for catestatin variants' inhibition of three nicotinic processes are plotted on logarithmic scales. The rank order of potencies for catestatin variants was the same for each process: Pro370Leu > wild-type > Gly364Ser > Arg374Gln. IC_{50} values correlated for secretion versus desensitization ($r = 0.997$), secretion versus transcription ($r = 0.999$), and desensitization versus transcription ($r = 0.999$).

(ANOVA $F = 0.071$, $p = 0.932$), and variant inhibitory potencies correlated well across processes (secretion versus signaling, $r = 0.986$; secretion versus desensitization, $r = 0.997$; secretion versus transcription, $r = 0.999$; desensitization versus transcription, $r = 0.999$). Hence, these four chromaffin cell nicotinic processes are likely to be mediated by similar (if not identical) combinations of neuronal nicotinic receptor α/β subunits, and the crucial amino acid residues within catestatin are likely to be similar across the four processes of nicotinic inhibition, at least for CGA residues 364, 370, and 374. In chromaffin cells, likely neuronal nicotinic subunit combinations include "ganglionic" type $(\alpha3)_2(\beta4)_3$ heteropentamers (McLane et al., 1990; Anonymous, 1995; Lopez et al., 1998). The present experiments cannot distinguish among these receptor target possibilities.

The time courses of our nicotinic process stimulation experiments ranged from 5 min (Na^+ cellular uptake; Fig. 4) to 30 min (secretion; Figs. 1–3 and 5–8) and finally to 18 h (transcription/translation; Fig. 9). Such experimental timing was governed by the cell biology of the process under investigation; i.e., coupled transcription/translation, to a luciferase reporter activity, requires many hours of agonist exposure. However, nicotinic cholinergic processes are also subject to time-dependent, agonist-induced desensitization (Mahata et al., 1999); indeed, for some nicotinic receptor isoforms, such as $(\alpha7)_5$ homopentamers, the time course for desensitization may be quite rapid (on the order of 1 s or less) (Lopez et al., 1998; Herrero et al., 2002). Thus, even though we clearly demonstrated substantial effects of the catestatin variants on nicotinic responses in three functional realms (Fig. 10; signal transduction, secretion, and transcription/translation), as well effects on agonist-induced desensitization (Fig. 8), at least some nicotinic processes could desensitize so rapidly (Lopez et al., 1998; Herrero et al., 2002) that we might be unable to define a catestatin influence on such events.

Although catestatin's initial inhibition of nicotinic cationic signaling (Fig. 4) diminished catecholamine release (Figs. 1–3 and 5–8), the peptide's blockade of agonist-induced desensitization (Fig. 8) ultimately enhanced catecholamine release. Thus catestatin's net effects on catecholamine secretion are likely to be dependent upon the prevailing degree of endogenous nicotinic cholinergic preganglionic tone to chromaffin cells and sympathetic axons; during circumstances of heightened sympa-

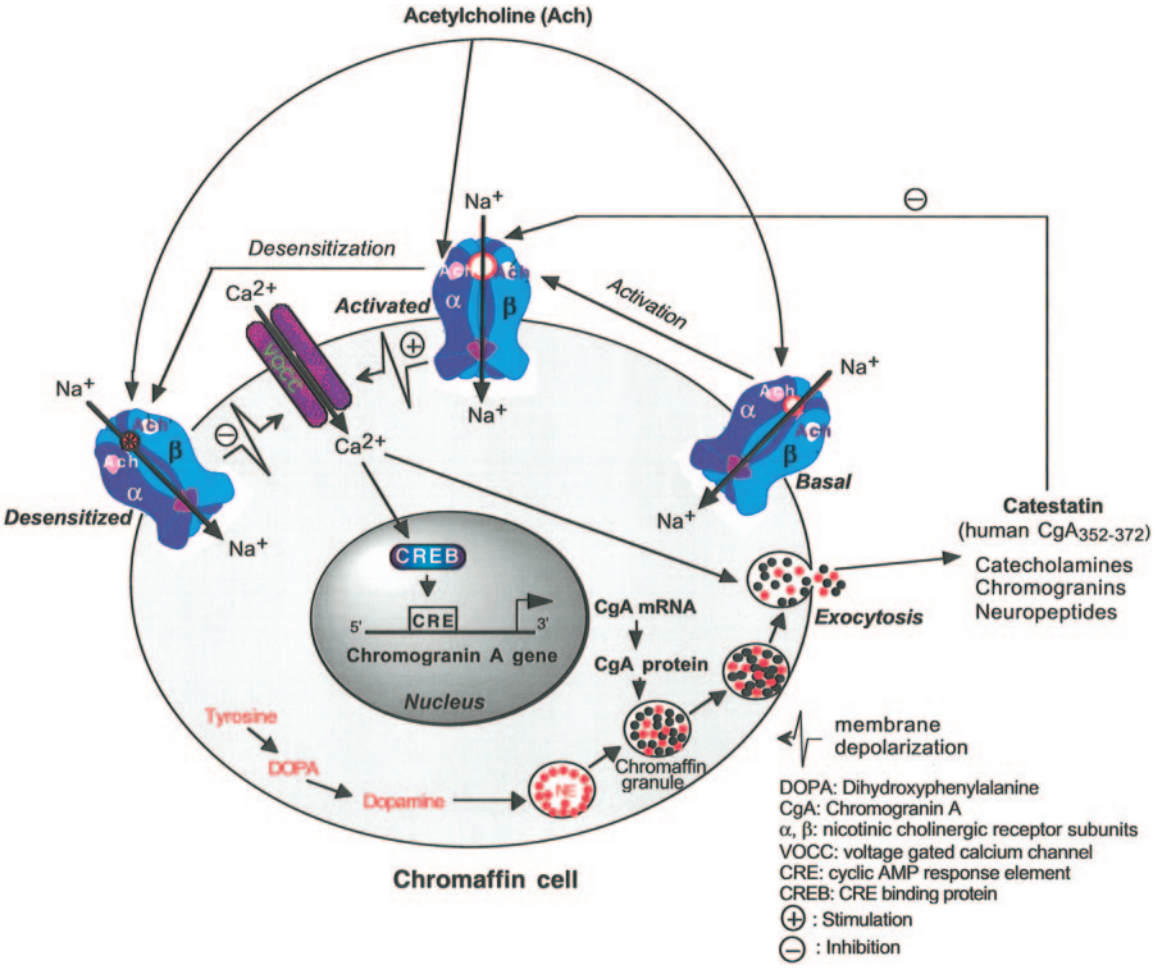

Fig. 11. Autocrine, negative feedback regulation of nicotinic cholinergic processes by catestatin variants. The diagram illustrates nicotinic cholinergic receptor activation in chromaffin cells by the endogenous agonist, acetylcholine (Ach), with subsequent mobilization of signal transduction [initially ligand-gated extracellular Na^+ entry; then Ca^{2+} entry through voltage operated calcium channels (VOCC)]. Further downstream activation of secretion and transcription ("stimulus transcription coupling") is also illustrated. Agonist stimulation of receptor activation (opening of the cation pore formed by the α and β subunits), followed by receptor desensitization (closing of the cation pore) is depicted. Finally, the actions of catestatin, coreleased by exocytosis with catecholamines, are shown on several nicotinic cholinergic processes: receptor activation, initial cationic signaling, subsequent signaling to secretion and transcription, and finally receptor desensitization.

thetic outflow, catestatin might actually function to sustain catecholamine release (Mahata et al., 1999).

Genetic Mechanisms: Dominant/Recessive Phenotypes. The minor allele frequencies for the catestatin variants range from 0.3–3.1% (Table 1), such that the ~4% of humans with such variants are likely to be heterozygotes (expressing one copy of the variant allele and one copy of the wild-type allele) in a randomly mating population at Hardy-Weinberg equilibrium (Falconer and Mackay, 1996); indeed, in the $n = 180$ persons subjected to CGA resequencing for SNP discovery, no homozygotes for the minor alleles were found.

What are the functional consequences of such heterozygosity? We attempted to answer this question by in vitro coexpression of the likely allelic combinations. In each case, the more active (more favorable/lower IC_{50}) allele seemed to exert phenotypic dominance over the less active allele (Fig. 6): Pro370Leu over wild type and wild type over either Gly364Ser or Arg374Gln. These results yield intriguing predictions for future in vivo studies of CGA genotype-to-phenotype effects.

References

Aardal S, Helle KB, Elsayed S, Reed RK, and Serck-Hanssen G (1993) Vasostatins, comprising the N-terminal domain of chromogranin A, suppress tension in isolated human blood vessel segments. *J Neuroendocrinol* **5:**405–412.

Anonymous (1995) 1995 Receptor and ion channel nomenclature supplement. *Trends Pharmacol Sci* **Suppl:**1–73.

Badio B and Daly JW (1994) Epibatidine, a potent analgetic and nicotinic agonist. *Mol Pharmacol* **45:**563–569.

Bartlett SF and Smith AD (1974) Adrenal chromaffin granules: isolation and disassembly. *Methods Enzymol* **31:**379–389.

DeQuattro V and Feng M (2002) The sympathetic nervous system: the muse of primary hypertension. *J Hum Hypertens* **16 Suppl 1:**S64–S69.

Falconer DS and Mackay TFC (1996) *Introduction to Quantitative Genetics.* Addison Wesley Longman, Harlow, UK.

Greene LA and Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci USA* **73:**2424–2428.

Herrero CJ, Ales E, Pintado AJ, Lopez MG, Garcia-Palomero E, Mahata SK, O'Connor DT, Garcia AG, and Montiel C (2002) Modulatory mechanism of the endogenous peptide catestatin on neuronal nicotinic acetylcholine receptors and exocytosis. *J Neurosci* **22:**377–388.

Lee JC, Taylor CV, Gaucher SP, Toneff T, Taupenot L, Yasothornsrikul S, Mahata SK, Sei C, Parmer RJ, Neveu JM, et al. (2003) Primary sequence characterization of catestatin intermediates and peptides defines proteolytic cleavage sites utilized for converting chromogranin a into active catestatin secreted from neuroendocrine chromaffin cells. *Biochemistry* **42:**6938–6946.

Li YF, Gong ZH, Cao JB, Wang HL, Luo ZP, and Li J (2003) Antidepressant-like effect of agmatine and its possible mechanism. *Eur J Pharmacol* **469:**81–88.

Lopez MG, Montiel C, Herrero CJ, Garcia-Palomero E, Mayorgas I, Hernandez-Guijo JM, Villarroya M, Olivares R, Gandia L, McIntosh JM, et al. (1998) Unmasking the functions of the chromaffin cell alpha7 nicotinic receptor by using short pulses of acetylcholine and selective blockers. *Proc Natl Acad Sci USA* **95:**14184–14189.

Mahapatra NR, Mahata M, O'Connor DT, and Mahata SK (2003) Secretin activation of chromogranin A gene transcription. Identification of the signaling pathways in cis and in trans. *J Biol Chem* **278:**19986–19994.

Mahata M, Mahapatra NR, O'Connor DT, and Mahata SK (2002) Chromaffin cell catecholamine secretion: bisindolylmaleimide compounds exhibit novel and potent antagonist effects at the nicotinic cholinergic receptor in pheochromocytoma cells. *Mol Pharmacol* **61:**1340–1347.

Mahata M, Mahata SK, Parmer RJ, and O'Connor DT (1996) Vesicular monoamine transport inhibitors. Novel action at calcium channels to prevent catecholamine secretion. *Hypertension* **28:**414–420.

Mahata SK, Mahapatra NR, Mahata M, Wang TC, Kennedy BP, Ziegler MG, and O'Connor DT (2003) 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. *J Biol Chem* **278:**32058–32067.

Mahata SK, Mahata M, Parmer RJ, and O'Connor DT (1999) Desensitization of catecholamine release: the novel catecholamine release-inhibitory peptide catestatin (chromogranin A344–364) acts at the receptor to prevent nicotinic cholinergic tolerance. *J Biol Chem* **274:**2920–2928.

Mahata SK, Mahata M, Wakade AR, and O'Connor DT (2000) Primary structure and function of the catecholamine release inhibitory peptide catestatin (chromogranin A344–364): identification of amino acid residues crucial for activity. *Mol Endocrinol* **14:**1525–1535.

Mahata SK, O'Connor DT, Mahata M, Yoo SH, Taupenot L, Wu H, Gill BM, and Parmer RJ (1997) Novel autocrine feedback control of catecholamine release. A discrete chromogranin A fragment is a noncompetitive nicotinic cholinergic antagonist. *J Clin Investig* **100:**1623–1633.

McLane KE, Wu XD, and Conti-Tronconi BM (1990) Identification of a brain acetylcholine receptor α subunit able to bind α-bungarotoxin. *J Biol Chem* **265:**9816–9824.

O'Connor DT, Frigon RP, and Sokoloff RL (1984) Human chromogranin A. Purification and characterization from catecholamine storage vesicles of human pheochromocytoma. *Hypertension* **6:**2–12.

O'Connor DT, Kailasam MT, Kennedy BP, Ziegler MG, Yanaihara N, and Parmer RJ (2002) Early decline in the catecholamine release-inhibitory peptide catestatin in humans at genetic risk of hypertension. *J Hypertens* **20:**1335–1345.

O'Connor DT, Takiyyuddin MA, Printz MP, Dinh TQ, Barbosa JA, Rozansky DJ, Mahata SK, Wu H, Kennedy BP, Ziegler MG, et al. (1999) Catecholamine storage vesicle protein expression in genetic hypertension. *Blood Press* **8:**285–295.

Preece NE, Nguyen M, Mahata M, Mahata SK, Mahapatra NR, Tsigelny I, and O'Connor DT (2004) Conformational preferences and activities of peptides from the catecholamine release-inhibitory (catestatin) region of chromogranin A. *Regul Pept* **118:**75–87.

Rumantir MS, Jennings GL, Lambert GW, Kaye DM, Seals DR, and Esler MD (2000) The 'adrenaline hypothesis' of hypertension revisited: evidence for adrenaline release from the heart of patients with essential hypertension. *J Hypertens* **18:**717–723.

Schober M, Howe PR, Sperk G, Fischer-Colbrie R, and Winkler H (1989) An increased pool of secretory hormones and peptides in adrenal medulla of stroke-prone spontaneously hypertensive rats. *Hypertension* **13:**469–474.

Takiyyuddin MA, Cervenka JH, Sullivan PA, Pandian MR, Parmer RJ, Barbosa JA, and O'Connor DT (1990) Is physiologic sympathoadrenal catecholamine release exocytotic in humans? *Circulation* **81:**185–195.

Takiyyuddin MA, De Nicola L, Gabbai FB, Dinh TQ, Kennedy B, Ziegler MG, Sabban EL, Parmer RJ, and O'Connor DT (1993) Catecholamine secretory vesicles. Augmented chromogranins and amines in secondary hypertension. *Hypertension* **21:**674–679.

Takiyyuddin MA, Parmer RJ, Kailasam MT, Cervenka JH, Kennedy B, Ziegler MG, Lin MC, Li J, Grim CE, Wright FA, et al. (1995) Chromogranin A in human hypertension. Influence of heredity. *Hypertension* **26:**213–220.

Tang K, Wu H, Mahata SK, Mahata M, Gill BM, Parmer RJ, and O'Connor DT (1997) Stimulus coupling to transcription versus secretion in pheochromocytoma cells. Convergent and divergent signal transduction pathways and the crucial roles for route of cytosolic calcium entry and protein kinase C. *J Clin Investig* **100:**1180–1192.

Tang K, Wu H, Mahata SK, and O'Connor DT (1998) A crucial role for the mitogen-activated protein kinase pathway in nicotinic cholinergic signaling to secretory protein transcription in pheochromocytoma cells. *Mol Pharmacol* **54:**59–69.

Tang K, Wu H, Mahata SK, Taupenot L, Rozansky DJ, Parmer RJ, and O'Connor DT (1996) Stimulus-transcription coupling in pheochromocytoma cells. Promoter region-specific activation of chromogranin A biosynthesis. *J Biol Chem* **271:**28382–28390.

Tatemoto K, Efendic S, Mutt V, Makk G, Feistner GJ, and Barchas JD (1986) Pancreastatin, a novel pancreatic peptide that inhibits insulin secretion. *Nature (Lond)* **324:**476–478.

Taupenot L, Mahata M, Mahata SK, and O'Connor DT (1999) Time-dependent effects of the neuropeptide PACAP on catecholamine secretion. Stimulation and desensitization. *Hypertension* **34:**1152–1162.

Taupenot L, Mahata SK, Wu H, and O'Connor DT (1998) Peptidergic activation of transcription and secretion in chromaffin cells. cis and trans signaling determinants of pituitary adenylyl cyclase-activating polypeptide (PACAP). *J Clin Investig* **101:**863–876.

Taylor CV, Taupenot L, Mahata SK, Mahata M, Wu H, Yasothornsrikul S, Toneff T, Caporale C, Jiang Q, Parmer RJ, et al. (2000) Formation of the catecholamine release-inhibitory peptide catestatin from chromogranin A. Determination of proteolytic cleavage sites in hormone storage granules. *J Biol Chem* **275:**22905–22915.

Tischler AS, Powers JF, and Alroy J (2004) Animal models of pheochromocytoma. *Histol Histopathol* **19:**883–895.

Tsigelny I, Mahata SK, Taupenot L, Preece NE, Mahata M, Khan I, Parmer RJ and O'Connor DT (1998) Mechanism of action of chromogranin A on catecholamine release: molecular modeling of the catestatin region reveals a b-strand/loop/b-strand structure secured by hydrophobic interactions and predictive of activity. *Regul Pept* **77:**43–53.

Wen G, Mahata SK, Cadman P, Mahata M, Ghosh S, Mahapatra NR, Rao F, Stridsberg M, Smith DW, Mahboubi P, Schork NJ, O'Connor DT and Hamilton BA (2004) Both rare and common polymorphisms contribute functional variation at CHGA, a regulator of catecholamine physiology. *Am J Hum Genet* **74:**197–207.

Winkler H and Fischer-Colbrie R (1992) The chromogranins A and B: the first 25 years and future perspectives. *Neuroscience* **49:**497–528.

Wu HJ, Rozansky DJ, Parmer RJ, Gill BM, and O'Connor DT (1991) Structure and function of the chromogranin A gene. Clues to evolution and tissue-specific expression. *J Biol Chem* **266:**13130–13134.

Address correspondence to: Dr. Sushil K. Mahata, University of California at San Diego and VASDHS (0838), 9500 Gilman Drive, San Diego, CA 92093-0838. E-mail: smahata@ucsd.edu