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Catestatin: A multifunctional peptide from chromogranin A

Sushil K. Mahata[*],
Manjula Mahata,
Maple M. Fung,
Daniel T. O'Connor

Department of Medicine (0838), University of California at San Diego, and Veterans Affairs San Diego Healthcare System, 9500 Gilman Drive, La Jolla, CA 92093-0838, USA

Abstract

In 1997, we identified a novel peptide, catestatin (CST: bovine chromogranin A $[CHGA]_{344–364}$: RSMRLSFRAR-GYGFRGPGLQL; human $CHGA_{352–372}$: SSMKLSFRARGYGFRGPGPQL), which is a potent inhibitor of nicotinic-cholinergic-stimulated catecholamine secretion. CST shows characteristic inhibitory effects on nicotinic cationic (Na^+, $Ca2^+$) signal transduction, which are specific to the neuronal nicotinic receptor. Utilizing systematic polymorphism discovery at the human *CHGA* locus we discovered three human variants of CST: $G^{364}S$, $P^{370}L$, and $R^{374}Q$ that showed differential potencies towards the inhibition of catecholamine secretion. In humans, CHGA is elevated and its processing to CST is diminished in hypertension. Diminished CST is observed not only in hypertensive individuals but also in the early-normotensive offspring of patients with hypertension, suggesting that an early deficiency of CST might play a pathogenic role in the subsequent development of the disease. Consistent with human findings, prevention of endogenous CST expression by targeted ablation (knockout) of the mouse *Chga* locus (*Chga*-KO) resulted in severe hypertension that can be "rescued" specifically by replacement of the CST peptide. CST acts directly on the heart to inhibit the inotropic and lusitropic properties of the rodent heart and also acts as a potent vasodilator in rats and humans. While the $G^{364}S$ CST variant caused profound changes in human autonomic activity and seemed to reduce the risk of developing hypertension, CST replacement rescued *Chga*-KO mice from dampened baroreflex sensitivity. In addition, CST has been shown to induce chemotaxis and acts as an antimicrobial as well as an antimalarial peptide. The present review summarizes these multiple actions of CST.

Keywords

Chromaffin; Chromogranin; Nicotine; Blood pressure; Chemotaxis; Innate immunity; Baroreflex sensitivity; Myocardial contractility

1. Introduction

Chromogranin A (CHGA (MIM 118910)), the index member of the chromogranin/secretogranin protein family, is a 48-kDa acidic polypeptide, which is the major protein

[*]Corresponding author. Tel.: +1 858 5528585x2637; fax: +1 858 6426425. smahata@ucsd.edu (S.K. Mahata).

found in the core of catecholamine storage vesicles of chromaffin cells and postganglionic sympathetic axons [1–4]. This protein is stored and released from the same secretory vesicles that contain catecholamines in chromaffin cells and noradrenergic neurons [5,6]. CHGA is required for the formation of catecholamine secretory vesicles in chromaffin cells and its expression may be sufficient to induce a regulated secretory system even in non-secretory cells [7]. Although initially detected in chromaffin granules, this protein was later found to be distributed ubiquitously in secretory vesicles of endocrine, neuroendocrine, and neuronal cells [1,8–10]. Because of the presence of 8–10 dibasic sites [11–15], CHGA also serves as a pro-hormone that gives rise to biologically active peptides such as the dysglycemic peptide pancreastatin ($hCHGA_{250–301}$) [16–18], the antimicrobial peptide prochromacin ($bCHGA_{79–431}$) [19], the vasodilator vasostatin ($hCHGA_{1–76}$) [20], and CST ($bCHGA_{344–364}$; $hCHGA_{352–372}$) that acts to inhibit catecholamine release [21]. CST also inhibits desensitization of catecholamine release induced by nicotine [22].

CHGA is overexpressed by chromaffin cells in rodent models of both genetic (spontaneously hypertensive rat) [23,24] and acquired (renovascular) [25] hypertension, and twin studies demonstrate the heritability of both plasma CHGA and CST concentration (44–60%) in humans [26,27]. In clinical practice, CHGA is used as a marker of pheochromocytomas [28–30], carcinoid tumors [31–33], neuroblastomas, neuroendocrine tumors, and neurodegerative diseases. Circulating CHGA levels are elevated in patients with chronic heart failure [34] and after acute myocardial infarction [35], which is partially supported by the finding of myocardial production of CHGA in humans with dilated and hypertrophic cardiomyopathy [36]. In addition, CHGA has been shown to increase in proportion to clinical severity and to be associated with prognosis in patients with both chronic and post-infarction heart failure [37]. In fact, CHGA is now claimed to be an independent predictor of long-term mortality and heart failure hospitalizations across the spectrum of acute coronary syndromes [38]. CHGA is also a good marker of sepsis and systemic inflammatory response syndrome [39,40] and an independent indicator of prognosis in critically ill nonsurgical patients [41]. In human essential (hereditary) hypertension, the plasma concentration of CST is diminished in not only established cases, but also in the early-normotensive offspring of patients with hypertension (FH^+) suggesting that an early deficiency of this catecholamine release-inhibitory peptide might play a pathogenic role in the subsequent development of the disease [42]. Consistent with human findings, prevention of endogenous CST expression by targeted ablation (knockout) of the mouse *Chga* locus (*Chga*-KO) results in severe hypertension that can be “rescued” specifically by replacement of the CST peptide [43].

CST causes vasodilation in rats [44] and humans [45], acting directly on the heart to regulate myocardial contractility and relaxation in the rodent heart [46] and to improve baroreflex sensitivity in *Chga*-KO mice [47]. CST also induces chemotaxis of human monocytes [48] and acts as an antibacterial peptide [49,50]. The present review focuses on these multiple actions of CST: effects on catecholamine secretion, cardiovascular physiology, chemotaxis and innate immunity.

2. Background and discovery of CST as the potent nicotinic-cholinergic antagonist

Although it was reported in 1988 that CHGA-proteolytic products inhibit catecholamine secretion from primary cultures of bovine chromaffin cells [51], the identity of the peptide remained elusive for about a decade. It was in 1997 when we identified the catecholamine release-inhibitory domain within CHGA by synthesizing 15 peptides (average length, 22 residues; range, 19–25 residues) spanning 78% of the length of the bovine CHGA (431 amino acid) and tested their effects (at 10 μM dose) on nicotine-evoked catecholamine secretion from PC12 cells [21]. Of the 15 peptides tested only one domain (bovine $CHGA_{344–364}$) was found to inhibit nicotine-evoked catecholamine secretion [21]. We coined the term “catestatin” to describe the catecholamine secretion inhibitory property of this peptide. In PC12 cells, CST showed an IC_{50} of 200 nM [21]. Given that the concentration of CHGA in chromaffin granules was ~2–4 mM and the concentration of chromaffin granule core contents in the extracellular space in the vicinity of the exocytotic pore was 10-fold lower than in the granule, the CHGA concentration was expected to be ~0.2–0.4 mM in the local extracellular space just after exocytosis. Therefore, our experimentally determined IC_{50} of ~200 nM in PC12 cells appeared to be physiologically relevant [21]. Similar results were obtained in bovine chromaffin cells, neurite-bearing (post-nerve growth factor treatment) PC12 cells [21] and in primary cultures of hippocampal neurons [52].

3. Proteolytic processing of CHGA to generate CST

Like the other pro-hormones, CHGA contains 8–10 dibasic residue sites, which are considered as potential sites for proteolytic cleavage [1]. Our first indication that CST was generated from CHGA came from the observation that low molecular weight chromaffin vesicle peptides, identified with an antiserum directed against synthetic CST, inhibited secretory activity [21]. Subsequently, we found extensive processing of CST in CHGA by evaluating CST radioimmunoassay of size-fractionated chromaffin granules. The major CST form identified by matrix-assisted laser desorption ionization (MALDI) mass spectrometry and confirmed by diagnostic M^{346} oxidation was bovine $CHGA_{332–364}$, which is flanked by dibasic sites (Fig. 1A–D) [53]. Of note, the preferred cleavage site for PC1 and PC2 pro-hormone convertases is at the COOH-terminal sides of paired basic residues [54–56]. We also detected human $CHGA_{340–372}$ as the major form in human pheochromocytoma chromaffin granules [53]. Subsequent studies identified secretion of CST ($bCHGA_{344–364}$: RSMRLSFRAR-GYGFRGPGLQL; calculated m/z = 2425.8) from primary cultures of bovine chromaffin cells in response to KCl depolarization [57]. Besides the well-established subtilisin-like serine proteases, we have found that in vitro digestion of recombinant CHGA with a serine protease plasmin generated a 14-amino acid peptide from the C-terminal end of CST ($CHGA_{360–373}$) [58–60], which showed nicotinic inhibition of catecholamine secretion (IC_{50} of ~2–3 μM). Because of the recent revelation that cysteine protease cathepsin L (CTSL) acts as a novel enzyme for proteolytic processing of neuropeptides [61–63], we first determined its localization within chromaffin granules/vesicles, and then identified active CST-region fragments ($CHGA_{360–373}$) by proteolytic cleavage of CHGA [64]. Of note, both

plasmin and CTSL were unable to cleave the variant CHGA-$P^{370}L$ to generate functional CST peptide ($CHGA_{360–373}$).

4. CST inhibition of catecholamine release

4.1. In vitro effects in PC12 and bovine chromaffin cells

Our initial studies with bovine CST ($bCHGA_{344–364}$) showed dose-dependent inhibition of nicotine-evoked catecholamine secretion from PC12 cells with an IC_{50} of ~0.2 μM [21]. Similar inhibition of nicotine-induced catecholamine secretion was seen in neurite-(NGF treatment for 5 days) bearing PC12 cells [65], primary cultures of bovine adrenal chromaffin cells [66] and rat hippocampal neurons [67]. In addition, the IC_{50} values for CST blockade in voltage-clamped oocytes expressing several combinations of neuronal nAChR subunits including α3β4, α3β2, α4β2, and α7 were found to be 0.3 μM for α7, 0.4 μM for both α3β2 and α3β4, and 1.7 μM for α4β2 receptors [68]. Testing N- and C-terminal, as well as bidirectional deletions, of CST in PC12 cells revealed that a completely active core sequence of CST is constituted by the 15 N-terminal amino acids of CST ($bCHGA_{344–358}$) [69]. Selective substitution of R by A implicated arginine residues at positions 351, 353 and 358 as crucial for inhibitory activity of CST [70], which were supported by our modeling [70] and NMR studies [71]. Further studies utilizing a series of single amino acid truncations or single residue substitutions by alanine, uncovered important roles of the following amino acids for their effects on profound suppression of nicotine-evoked catecholamine secretion compared to wild-type CST: $L^{348}A$ (by ~9.6-fold), and $F^{350}A$ (by ~12-fold), $R^{351}A$ (by ~15.7-fold), $R^{353}A$ (by ~17.8-fold) and $R^{358}A$ (by ~20.7-fold) [69] (Fig. 2A & B).

We discovered three human variants of CST: $G^{364}S$, $P^{370}L$, and $R^{374}Q$ from resequencing CHGA in 180 individuals ($2n$ = 360 chromosomes) (Table 1). Up to ~4% of the human chromosomes encoded one of these CST amino acid variants. Testing these variants on the inhibition of nicotine-induced catecholamine secretion revealed the following rank order of potency: $P^{370}L$ (IC_{50} 0.37 ± 0.03 μM)>wild-type (IC_{50} 0.82±0.02 μM)>$G^{364}S$ (IC_{50} 3.65 ± 0.11 μM) (Fig. 3) [72,73]. A decrease in potency was paralleled by a decline in the Hill slope, suggesting negative cooperativity at higher doses might underlie the observed loss of potency [73].

CST inhibition of catecholamine secretion was found to be specific to stimulation by nicotine. CST was unable to inhibit catecholamine secretion when it was induced by secretagogues that bypass the nicotinic-cholinergic receptor (nAChR), including membrane depolarization (by 55 mM KCl) [21] to open voltage-gated calcium channels, an alkaline earth (2 mM $BaCl_2$) to block cell surface K^+ channels and thereby depolarize the cell membrane [21], and a Ca^{2+} ionophore (1 μM ionomycin) to admit extracellular Ca^{2+} to the cytosol [21] as well as by secretagogues that target different receptors to the nAChR, such as ATP (100 μM) acting on the P_{2x} purinergic receptor [21], or PACAP (0.2 μM) acting on the PAC1 G-protein-coupled receptor [21,74]. CST inhibition of catecholamine secretion was found to be at the very 1st step in nicotinic cationic signal transduction i.e., at the level of Na^+ uptake followed by the inhibition of Ca^{2+} uptake. Since CST inhibition of catecholamine secretion remained unaltered by log_{10}-ascending doses of nicotine (10–1000

μM) we concluded that CST antagonism of nicotine action was noncompetitive in nature [21].

4.2. Ex vivo effect in the superfused rat adrenal gland

The superfused rat adrenal gland model has been widely used by Wakade's group to distinguish contribution of cholinergic (e.g., acetylcholine) and peptidergic (vasoactive intestinal peptide and pituitary adenylyl cyclase activating polypeptide) secretagogues in evoking catecholamine secretion [75–77]. In the superfused rat adrenal gland, we found that CST caused the inhibition of catecholamine secretion induced by both the stimulation of the splanchnic nerve (10 Hz, 30 sec) and by nicotinic-cholinergic agonists (acetylcholine or nicotine), although the inhibition was most efficient (80%) for secretion caused by nicotine itself (Fig. 4A) [69]. Since nerve stimulation causes release of acetylcholine that may trigger catecholamine secretion by activating both muscarinic and nicotinic receptors in the rodent [78], the finding is consistent with the idea that CST is selective for the nicotinic-cholinergic mechanism [21,69].

4.3. In vivo effect in mice

Direct activation of nAChR by nicotine (2.5 mg/kg, IP) in transgenic mice (*Chga* promoter driving expression of the luciferase gene) caused an acute (30 min) 2.7-fold release of catecholamines (norepinephrine and epinephrine) into the bloodstream [79]. Pretreatment with CST (2.5 mg/kg, IP) resulted in 80% inhibition of nicotine-induced catecholamine secretion (Fig. 4B), indicating that this peptide can act in vivo as a nicotinic antagonist. This in vivo finding extends the significance of our in vitro studies of nicotinic signaling to catecholamine secretion [21,22,69] and establishes a fundamental role for CHGA and its CST fragment at the nexus of nicotinic-cholinergic signaling.

5. CST inhibition of desensitization of catecholamine release

Nicotinic receptors undergo desensitization upon prolonged or repeated exposure to the agonist [22]. CST was found to inhibit the nicotine-induced desensitization of catecholamine release with an IC_{50} of ~0.28 μM (Fig. 5) [22]. As with the inhibition of nicotine-evoked secretion of catecholamines by CST, the inhibition of desensitization by CST was found to be specific to nAChR activation and non-competitive in nature with respect to the agonist [22]. Prior nicotinic desensitization caused an 82% diminution of $^{22}Na^{+}$ uptake that was markedly inhibited by CST, with an IC_{50} ~0.28 μM (Fig. 5). We believe that the CST blockade of nicotinic desensitization of catecholamine release may be advantageous to an organism, particularly during stress when CST might sustain catecholamine release to counteract with the stress situation.

6. CST modulation of transcription of *Chga* gene

In cultured chromaffin cells *in vitro*, exocytotic stimuli promoted the resynthesis of newly released catecholamine storage vesicle contents, a process known as "stimulus–secretion–synthesis coupling" or "stimulus–transcription coupling" [80,81]. We sought to determine whether this phenomenon occurs in vivo. Treatment with nicotine caused a ~2-fold increase

in the expression of the *Chga*/luciferase transgene, confirming the phenomenon in vivo, and this increase was inhibited >90% by the intraperitoneal injection of CST [79]. This establishes an entirely new role for CST on gene expression *in vivo*.

7. CST stimulation of histamine release from mast cells

Because of the potent vasodilator action of CST in rats [44] we tested whether CST can induce the release of the vasodilator histamine from mast cells. The most active N-terminal domain of CST (bCHGA$_{344–358}$: RSMRLSFRARGYGFR) caused a concentration-dependent (0.01–5 μM) release of histamine from peritoneal and pleural mast cells [82]. CST was found to be the most potent activator of histamine release than the wasp venom mastoparan and the neuropeptide substance P. Since CST-evoked histamine release was suppressed by the pertussis toxin we suggested involvement of a G_i subunit in CST signaling to histamine release that is distinct from the mechanism of inhibition of catecholamine release from chromaffin cells as described above.

8. CST induction of chemotaxis and the underlying signaling cascade

CHGA, an important constituent of the plaques in Alzheimer's disease [83,84], activates monocyte-derived microglia that invades and surrounds the plaques [85–87]. Based on these findings, we reasoned that CST would regulate monocyte migration. Consistent with our hypothesis we found that CST caused a dose-dependent induction of chemotaxis in human monocytes, exerting its maximal effect at 1 nM, which is comparable to the established formylated chemoattractant (fMLP) [48]. At the receptor level, CST acts through a tyrosine kinase and a G-protein-coupled receptor involving sphingosine 1-phosphate. At the post-receptor signaling pathway level, CST signals through phosphoinositide 3-kinase, nitric oxide and mitogen activated protein kinase dependent pathways. The evaluation of CST effects in animal models of inflammation or Alzheimer's disease is crucial to determine the biological relevance of the chemotactic effect of CST.

9. CST action on innate immunity

The innate immunity refers to the inborn system of first defense against microorganisms. It is triggered by a range of natural cationic peptides isolated from insect lymph, frog skin, mammalian neutrophil granules and plants, and several CHGA peptides, including vasostatin-I [88]and prochromacin [89]. Because of the highly cationic nature of CST, a characteristic feature of the antibacterial compound, we reasoned that CST would act as an antibacterial peptide. The N-terminal domain of CST (CHGA$_{344–358}$), containing an arginine-rich amphiphilic domain, inhibited the growth of Gram-positive and Gram-negative bacteria, a variety of filamentous fungi and several forms of yeasts [25], without showing any hemolytic activity. This peptide rapidly passes through the cell membrane, accumulates in the inner part of the cells and possibly acts on intracellular targets. By using Western blot with a specific antibody we have also identified several CST-containing fragments (hCHGA$_{340–394}$) in the leukotoxin class S Panton-Valentine (2.3 nM) and leukotoxin class F Panton-Valentine (0.6 nM)-stimulated secretion medium of human polymorphonuclear neutrophils (PMN) [49]. Recently, it has been shown by Metz-Boutigue's group that CST

can penetrate into PMN, which basically qualifies CST to be a new member of the cell penetrating peptide family. In addition, they have shown that CST can penetrate into PMNs by a calmodulin-regulated calcium independent phospholipase A2 pathway [39]. Subsequently, we have detected CHGA in keratinocytes and demonstrated its processing to CST in the human skin, penetrating through the human epidermis and exhibiting inhibitory potencies against skin pathogens [102]. CST expression in the murine skin was found to have increased in response to injury and infection, which showed a potential for increased protection against infection [50]. These findings demonstrate a direct link between the neuroendocrine and immune systems.

Because of the inhibitory effects of antimicrobial peptides such as scorpine, magainin 2, cecropin B, defensin, and dermaseptin S3 and S4 on the inhibition of the growth of the malarial parasite [90–93], Metz-Boutigue's and Candolfi's groups recently tested *in vitro* the effects of CST on the growth of *Plasmodium falciparum* and found that CST acts as a potent inhibitor of the chroloquine-sensitive strain of *P. falciparum* 3D7, the chloroquine-resistant strain 7G8 as well as the multidrug-resistant strain W2 [94]. It is believed that CST exerts antimalarial activity possibly by inactivating plasmepsins, the aspartic proteases that are involved in the degradation of the host cell hemoglobin, providing nutrients for the growth of the malarial parasite [95]. They identified that the N-terminal serine residue in hCST is essential for maximal inhibition of growth of the malarial parasite. Measurement of plasma CST concentration in patients with malarial infection would establish a very important function of CST and may establish CST as a new player in protecting humans against malarial disease.

10. CST effect on cardiovascular system

10.1. Vasodilator effect of CST in the rat and human

Because of the potent catecholamine release-inhibitory effect of CST, we tested whether CST exerts effects on the cardiovascular system. Intravenous administration of CST to rats reduced pressor responses to the activation of sympathetic outflow by electrical (7.5 V 20 Hz) stimulation (Fig. 6) [44]. The CST effect persisted even after adrenergic (α [phenoxybenzamine, 20 mg/kg IP] plus β [propranolol, 2 mg/kg IV]) blockade [44]. Since the vasodepressor effect of CST was blocked by a histamine H_1 receptor antagonist (hydroxyzine, 5 mg/kg IV) and CST elevated endogenous circulating histamine 21-fold coupled with exogenous histamine mimicking the vasodepressor actions of CST, we concluded that CST is a potent vasodilator *in vivo* whose actions appear to be mediated, at least in part, by histamine release and action at H_1 receptors.

We have also evaluated the potential vasodilator effect of CST in 18 healthy human subjects (male and female) by infusing CST to achieve target concentrations of ~50, ~500, and ~5000 nM into dorsal hand veins without systemic counter-regulation, after pharmacological venoconstriction with phenylephrine. CST caused dose-dependent vasodilation predominantly in female subjects after phenylephrine-induced preconstriction to 69% (Fig. 7). Of note, the EC_{50} (~30 nM) for vasodilation induced by CST was the same order of magnitude to circulating endogenous CST (4.4 nM) [45]. We also found that despite low CHGA precursor concentrations, female subjects had higher plasma CST levels

than males, which reflects increased processing of CHGA-to-CST. These findings indicate that CST may contribute to the regulation of endogenous vascular tone and influence the complex predisposition to hypertension.

10.2. CST actions on lowering of blood pressure in rodents and humans

Hypertension is a complex trait with an ill-defined genetic predisposition, in which adrenergic mechanisms seem to be involved even at the early stages. Since excess sympathetic activity is implicated in causing hypertension, and alterations in sympathetic responses may occur in normotensive relatives of patients even prior to the onset of hypertension, it is expected that the CST mechanism might be altered in hypertension or in individuals at risk for the development of hypertension. Consistent with this hypothesis, we found that the plasma concentration of CST is diminished not only in established cases of essential (hereditary) hypertension, but also in the still-normotensive offspring of patients with hypertension (FH^+) [42]. This indicates that an early deficiency in CST might play a pathogenic role in the subsequent development of hypertension, suggesting a pathophysiologic mechanism linking CST to hypertension.

Consistent with the human findings, we detected high blood pressure (BP) and higher plasma catecholamines in mice after targeted ablation of the *Chga* gene (*Chga*-KO). Of note, the elevated BP was rescued by insertion of the human *CHGA* gene in the *Chga* null background [43], consistent with a hypotensive effect of CHGA. Furthermore, CST replacement rescued *Chga*-KO mice from the high resting BP (Fig. 8A) and plasma catecholamines. We have not yet determined whether this effect of CST was secondary to a histamine release from mast cells, as we have seen in our studies in the rat. Immobilization stress in telemetered mice caused increments in SBP and HR in both WT and *Chga*-KO mice, with higher maxima but blunted increments in the KO state [47]. CST replacement selectively diminished stress-induced increments in BP and HR in KO mice, implicating CST as an antihypertensive peptide even in stressful conditions. CST administration (30 min) rescued *Chga*-KO mice from higher plasma catecholamine, indicating that the CST restoration of elevated BP in *Chga*-KO mice [43] likely resulted from CST inhibition of catecholamine secretion from chromaffin cells.

To establish a functional role for CST in the central nervous system, Gaede et al. tested the effects of CST in the spinal cord by intrathecal injection of CST, in conjunction with nicotine and isoproterenol in the anesthetized rat. CST attenuated the hypotensive effect of isoproterenol and the hypertensive effect of nicotine on mean arterial pressure, splanchnic sympathetic nerve activity, and heart rate [96]. These findings have led the authors to conclude that CST antagonizes both central nicotinic acetylcholine receptors and β-adrenoceptors that are involved in cardiovascular regulation *in vivo*.

Based on the above findings we can state that CST regulates BP by acting as an inhibitor of peripheral [43,45] as well as central [96] nicotinic-cholinergic receptors and β-adrenoceptors [46,96]. We are yet to ascertain how CST modulates activities in both the nicotinic-cholinergic and adrenoceptor. However, our unpublished observations seem to suggest that CST can regulate BP by elevating reactive oxygen species as well as lipid peroxidation and depletion of nitric oxide (Gayen JR et al. unpublished observation).

10.3. Direct cardiovascular effects of CST

We have found that circulating levels of CST decrease in patients with essential hypertension [42] and targeted ablation of the *Chga* gene in mice increases BP, which can be "rescued" by replacement with CST [43], indicating a direct role of CST in preventing hypertension. This profound vasoreactivity prompted us to test the direct cardiovascular effects of CST on myocardial and coronary functions. In the Langendorff-perfused rat heart, CST dose-dependently increased heart rate and coronary pressure and decreased left ventricular pressure, and both positive and negative *LVdP/dt*. CST also abolished isoproterenol-induced positive inotropism and lusitropism [46]. In addition, CST inhibited endothelin-1-induced positive inotropism and coronary constriction. Signaling studies indicate that CST acts through β_2-ARs-Gi/o protein-NO-cGMP signaling pathways to exert its cardiosuppressive effect. Thus, in addition to its important role in the control of BP, CST is now emerging as a peptide that has direct cardiovascular actions under both basal and stimulated conditions, suggesting that the negative inotropism and lusitropism of CST may be important components of its hypotensive action.

In the hearts of homeotherms (e.g., mammals), the coronary vascular endothelium and endocardial endothelium act in concert to modulate humorally myocardial activity, making the contribution of the endocardial endothelium in the paracrine regulation of myocardial function difficult to define [97]. In contrast, in the avascular frog heart endocardial endothelium is the only barrier between the superfusing blood and the subjacent myocardial microenvironment and is therefore a unique model to analyze its autocrine/paracrine role in the transduction of blood-borne endoluminal chemical stimuli, which can target the myocardium [98]. Therefore, to delineate CST's direct myocardiotropic action, we used the avascular frog heart as a bioassay where CST dose-dependently decreased stroke volume and stroke work, with a threshold concentration of 11 nM, which is comparable to the in vivo level of the peptide (~2–4 nM) [99]. In addition, CST inhibited the positive inotropic effect induced by isoproterenol or endothelin-1.

10.4. CST regulation of autonomic function

Cardiovascular performance is controlled by the autonomic nervous system (ANS). Beat-to-beat fluctuation in the heart rate (HR) is a balanced consequence of ANS tone to the heart, both sympathetic (increasing HR) and parasympathetic (decreasing HR). We found that humans with a genetic variation in the CST region, particularly $G^{364}S$, displayed alterations in baroreceptor function, both parasympathetic and sympathetic. The G/S heterozygotes displayed an increased baroreceptor slope during upward and downward deflections (by ~47 and ~44%, respectively), an increased cardiac parasympathetic index (by ~2.4-fold), and a decreased cardiac sympathetic index (by ~26%) when compared with the G/G homozygotes. This CST variant seems to reduce the risk of developing hypertension, especially in men [52].

Abnormalities in baroreflex sensitivity (BRS) in experimental [100,101] and human hypertension have been demonstrated, with hypertensive subjects exhibiting diminished BRS compared with their normotensive counterparts [102–104]. Additionally, the family history of hypertension is associated with lower BRS in both the normotensive and

hypertensive offspring [105]. Since genetic variation at the human *CHGA* locus results in alterations in BP in the population, and that targeted ablation of the mouse *Chga* locus results in profound hypertension [43], we studied potential mechanisms of such changes in the ANS, using physiological, biochemical, and pharmacological probes. Consistent with hypertensive subjects, the BRS slope in *Chga*-KO mice was decreased by ~3-fold in response to reflex bradycardia caused by phenylephrine-induced hypertension and reflex tachycardia caused by sodium nitroprusside-induced hypotension (Fig. 8B) [47]. In addition, the set point was found to have increased in *Chga*-KO mice. Of note, CST replacement restored the dampened BRS slope in KO mice Fig. 8B, indicating that CST resets the entire ANS reflex arc to restore normal cardiovascular function. To probe the relative roles of endogenous/basal sympathetic versus parasympathetic tone in the control of BP and HR, we employed the muscarinic-cholinergic antagonist atropine or the beta-adrenergic antagonist propranolol where HR and BP responses to each antagonist were found to have exaggerated in *Chga*-KO animals. These findings may be attributable to either diminished baroreceptor function, or heightened outflow from both the parasympathetic and sympathetic branches, or both. Since the CHGA and CST mechanisms are altered in human hypertension, our results in this experimental animal model may provide insight into the pathogenesis of this common human disorder.

11. Conclusions and perspectives

CST ($bCHGA_{344–364}$; $hCHGA_{352–372}$) is now established to act as a potent inhibitor of nicotine-evoked catecholamine secretion (IC_{50} ~0.2–0.4 μM) in three preparations: *in vitro* in cultured chromaffin cells, *ex vivo* in the superfused rat adrenal gland and *in vivo* in mice. Thus, CST represents a novel, autocrine homeostatic (negative-feedback) mechanism controlling catecholamine release from chromaffin cells and noradrenergic neurons (Fig. 9). CST inhibition of desensitization of catecholamine release might be advantageous to an organism during stress, when the peptide might act to sustain catecholamine release. These basic studies on the mechanisms of action of CST *in vitro* and *in vivo* are given a physiological perspective with the observations in humans of reduced plasma CST levels associated with an augmented risk of developing hypertension and increased pressor responses to environmental stressors. The results with human CST variants indicate the possibilities for inter-individual variations in human nicotinic signaling as the human carriers of ^{364}S display profound alterations in autonomic activity in both the parasympathetic and sympathetic branches, which in turn may protect the 364Ser carrier against the future development of hypertension, especially in males. The vasodilatory effects of CST in the human hand vein, especially in females indicate that CST may contribute to sex differences in endogenous vascular tone and influence the complex predisposition to hypertension. Because of the potent inhibition of the inotropic and lusitropic properties of the rodent heart, CST is now considered as a novel cardiac modulator, which could protect the heart against excessive sympathetic drive such as that seen in hypertensive cardiomyopathy (Fig. 9). Restoration (elevation) of BRS sensitivity after exogenous CST in *Chga*-KO mice indicates that CST resets the entire autonomic nervous reflex arc to restore normal cardiovascular function. Evaluation of CST effects in animal models of inflammation or Alzheimer’s disease is crucial to determine the biological relevance

of the chemotactic effect of CST. The antimicrobial function of CST against a wide assortment of skin pathogens and its upregulation upon skin injury provides a direct link between the antimocrobial defense of the skin and the neuroendocrine peptide CST. CST is thus emerging as a very important peptide regulating multiple functions and bears all the potentials to be a therapeutic agent to treat multiple diseases like hypertension, cardiomyopathy, inflammation, malaria and skin infection.

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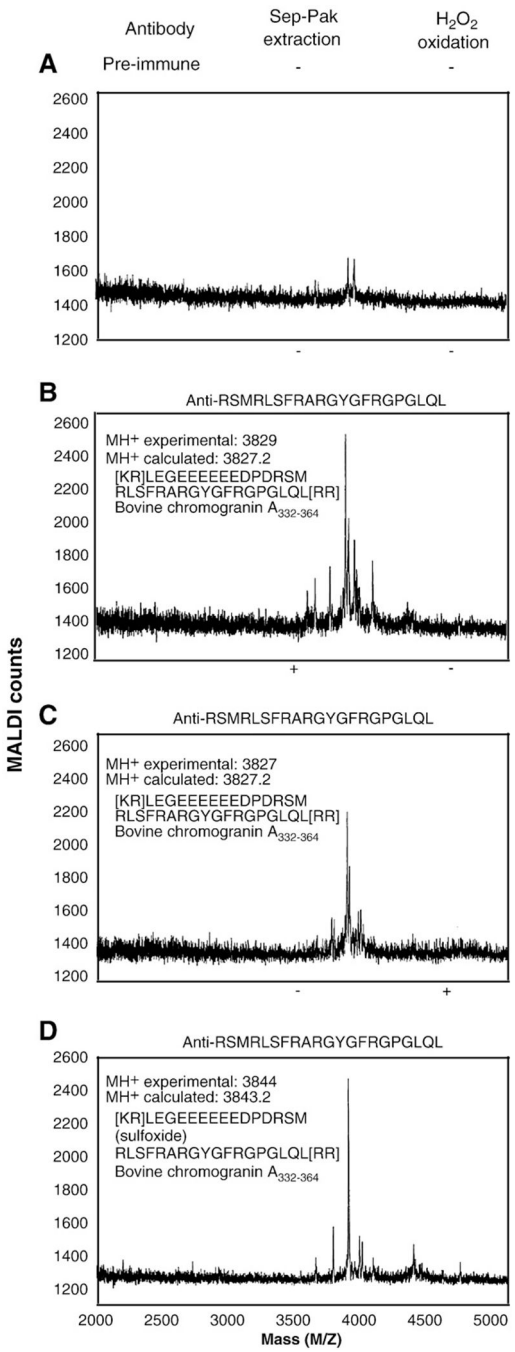

Fig. 1.

Identification of CST in immunoprecipitated bovine adrenal medullary chromaffin granules. Aliquots (200 ml) of the low molecular weight chromaffin granule peptides (devoid of full length CHGA) separated by gel filtration were immunoprecipitated (20 ml of anti-bovine CST antiserum) and then subjected to MALDI mass spectrometry (1–2 ml). (A) Immunoprecipitation by preimmune serum. (B) Immunoprecipitation by rabbit anti-bovine CST antiserum. (C) Immunoprecipitation by rabbit anti-bovine CST antiserum, followed by adsorption and elution from a C-18 (Sep-Pak) cartridge. (D) Immunoprecipitation by rabbit

anti-bovine CST after M^{346} oxidation by 10 μM H_2O_2 (reproduced with permission from The American Society for Biochemistry and Molecular Biology).

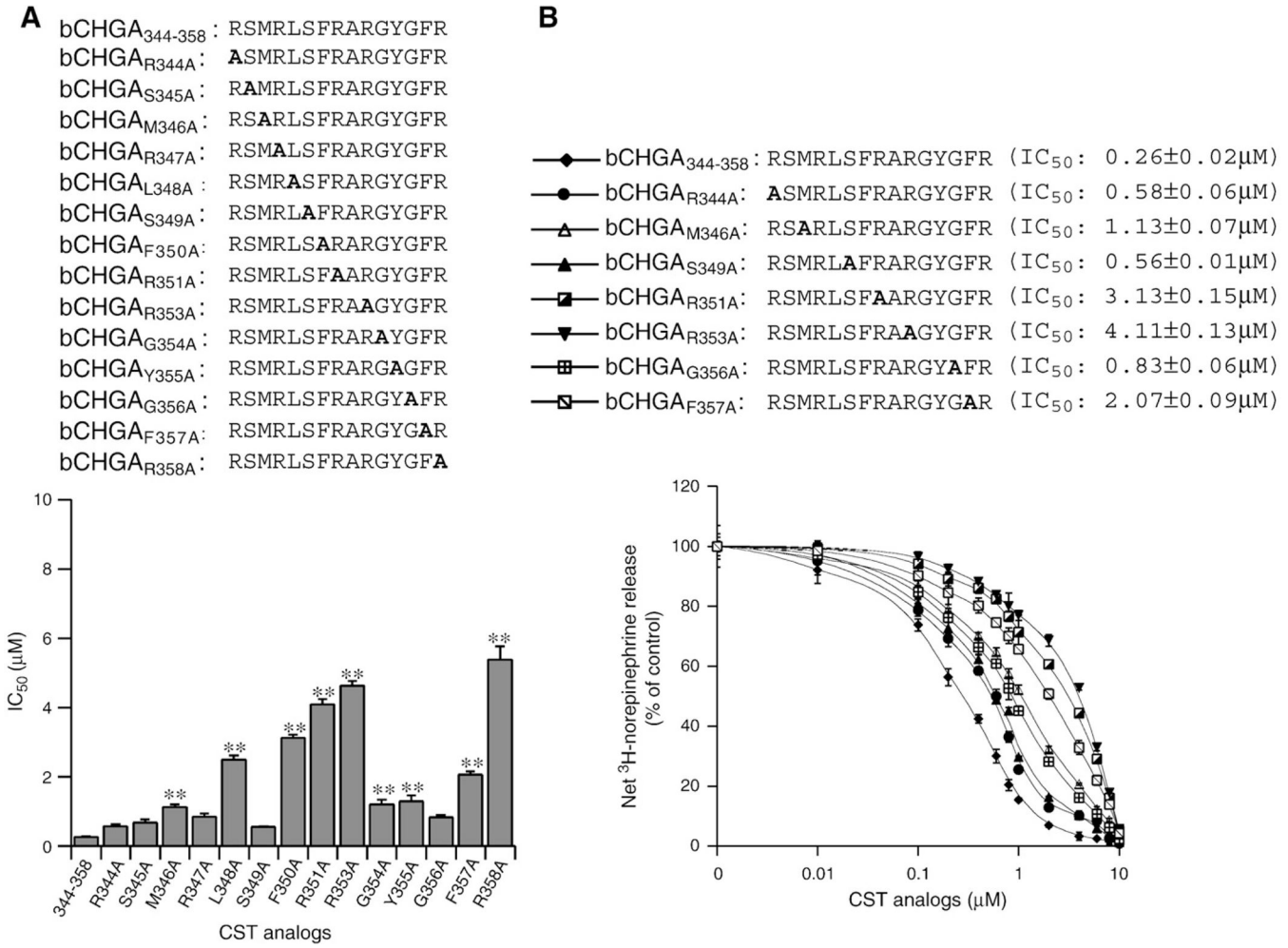

Fig. 2.

Identification of crucial amino acids in the active core of CST for the inhibition of catecholamine release. PC12 cells prelabeled with [^{3}H]L-norepinephrine were incubated with 60 μM nicotine, with or without logarithmically ascending doses (0.01 to 10 μM) of bovine CST for 30 min. (A). Effect of alanine substitution of individual amino acids. Amino acids preceding the numbers 344–358 represent the particular amino acid (and its position) substituted by alanine. For example, in bCHGA$_{R344A}$, R^{344} is substituted by A. Alanine substitutions are also shown in bold letters. (B). Graphic comparison of crucial amino acids in the active CST core (bovine CHGA$_{344–358}$) sequence for blockade of nicotinic-cholinergic-stimulated catecholamine release. Control (100%) release is that in the presence of nicotine (60 μM) stimulation alone. Results are shown as the mean ± SEM. IC$_{50}$ values of each peptide for the inhibition of secretion are given in parentheses. bCHGA, bovine chromogranin A. (Reproduced with permission from The Endocrine Society).

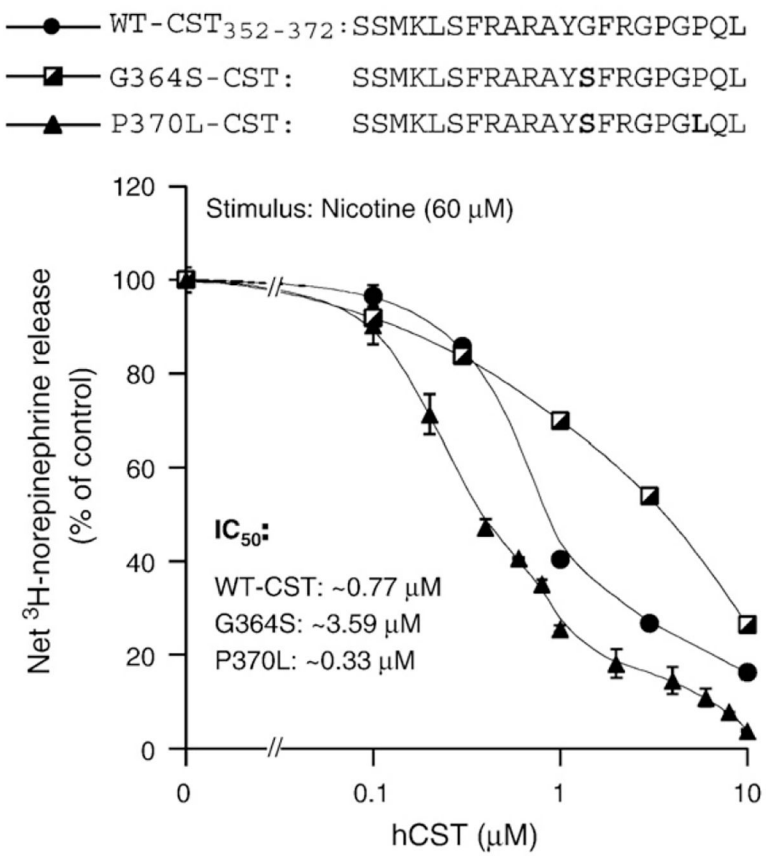

Fig. 3.

Altered efficacy of nicotinic inhibition by naturally occurring human CST variant peptides. PC12 cells prelabeled with [^{3}H]L-norepinephrine were incubated with 60 μM nicotine, with or without bovine CST (0.01 to 10 μM) for 30 min. Control (100%) release is that in the presence of nicotine (60 μM) stimulation alone. Results are shown as the mean ± SEM (reproduced with permission from The University of Chicago Press).

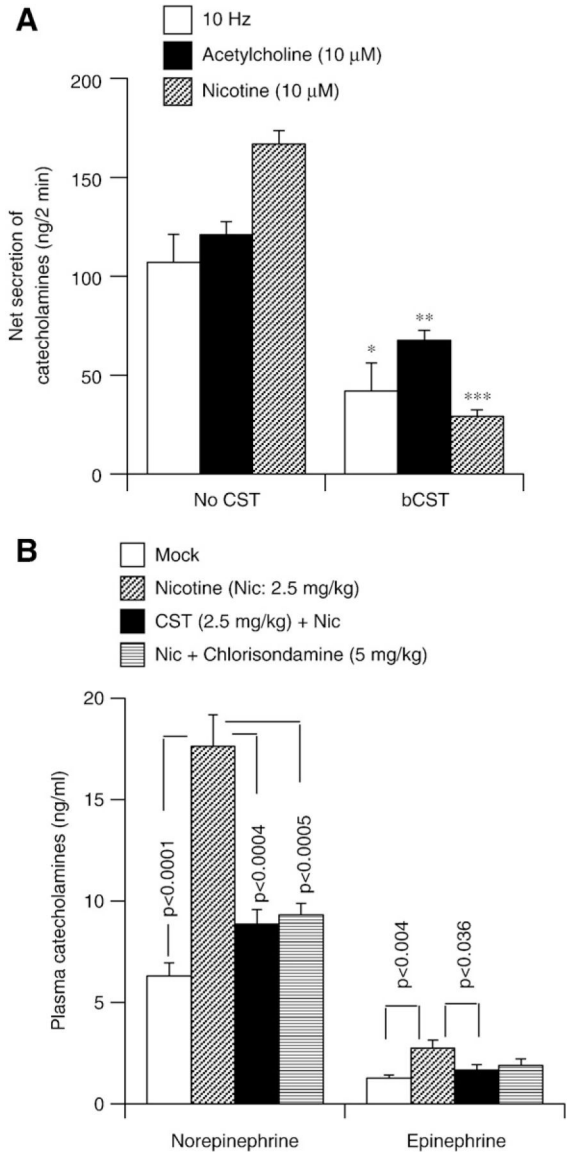

Fig. 4.

CST effects on catecholamine release ex vivo from the superfused rat adrenal gland and *in vivo* from the mouse adrenal gland. (A). Catecholamine secretion from superfused rat adrenal glands was induced by electrical stimulation of the splanchnic nerve (10 Hz, 30 s), acetylcholine (10 μM, 2 min), and nicotine (10 mM, 2 min), and compared to basal secretion. Perfusates were collected for 2 min for catecholamine assay. Experiments were conducted on 3 different days, and the results were averaged (mean ± SEM) after subtraction of basal (unstimulated) release. *, $p<0.03$; **, $p<0.003$; ***, $p<0.0001$ (stimulation with CST or without [no CST]) (reproduced with permission from The Endocrine Society). (B). Catecholamine release by nicotinic-cholinergic stimulation and blockade by nicotinic-cholinergic antagonists, including CST. The sympathoadrenal system was activated by the nicotinic-cholinergic agonist nicotine(2.5 mg/kg intraperitoneally)for comparison with vehicle alone (mock). Animals were pretreated 30 min prior to nicotine

or vehicle alone (mock) or with nicotinic-cholinergic antagonists (either the classical antagonist chlorisondamine 5 mg/kg intraperitoneally, or the novel peptide antagonist CST, 2.5 mg/kg intraperitoneally) to achieve an extracellular target concentration of ~4 μM. 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 determination of plasma catecholamines. Results are shown as mean ± S.E (reproduced with permission from The American Society for Biochemistry and Molecular Biology).

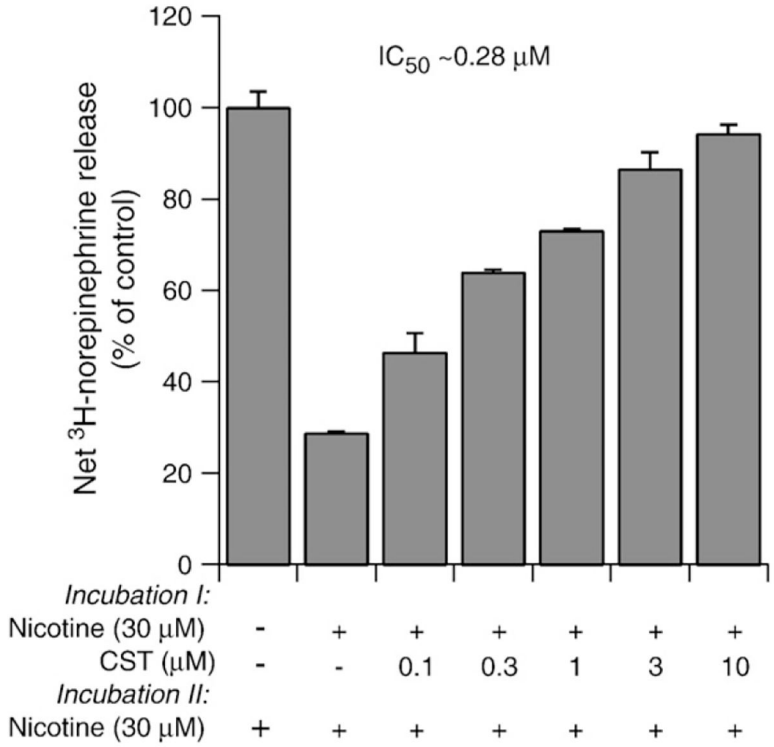

Fig. 5.

CST inhibition of desensitization of catecholamine release. L-[^{3}H]-norepinephrine preloaded cells were treated with the nicotinic-cholinergic agonist nicotine (30 μM) either alone or in combination with logarithmically ascending doses (0.01 to 10 μM) of bovine CST analogs or substance P (0.1 to 10 μM) for 10 min (incubation I), washed twice (6 min each), and rechallenged with nicotine (10 μM) for 10 min (incubation II) before measurement of norepinephrine secretion. Control cells received nicotine only in incubation II (reproduced with permission from The American Society for Biochemistry and Molecular Biology).

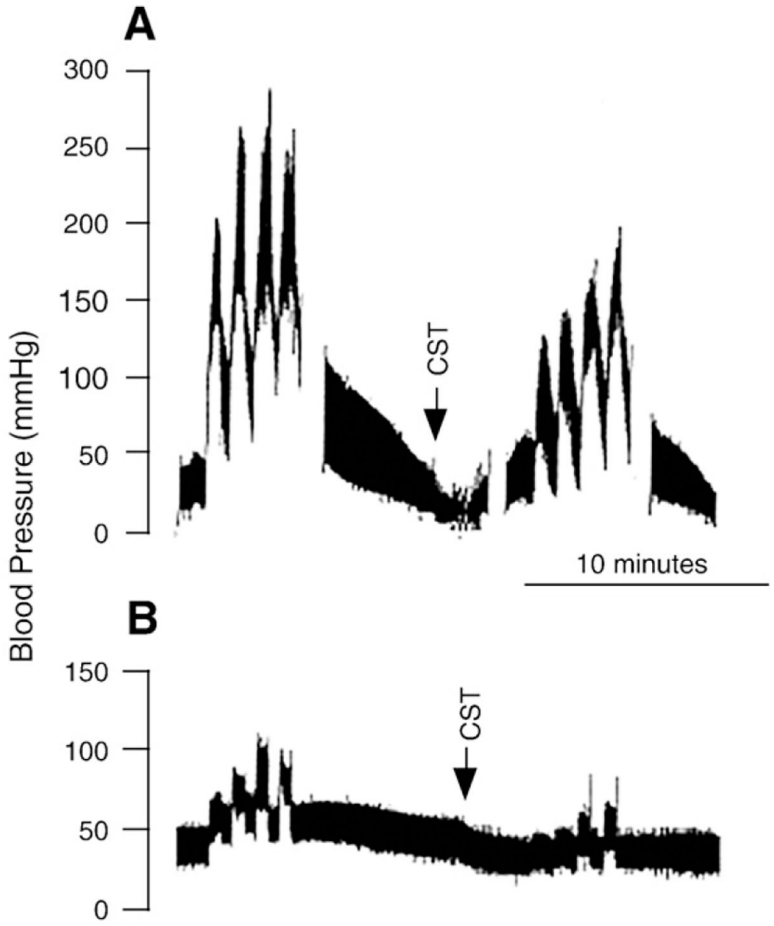

Fig. 6.

CST effect on BP in vivo in rat. Typical BP response during 7.5 V 20 Hz stimulation of the sympathetic nervous system of a pithed rat, before and after treatment with intravenous CST(0.3 μmol), without (A), or with (B) prior adrenergic blockade by propranolol (2 mg/kg) and phenoxybenzamine (20 mg/kg).

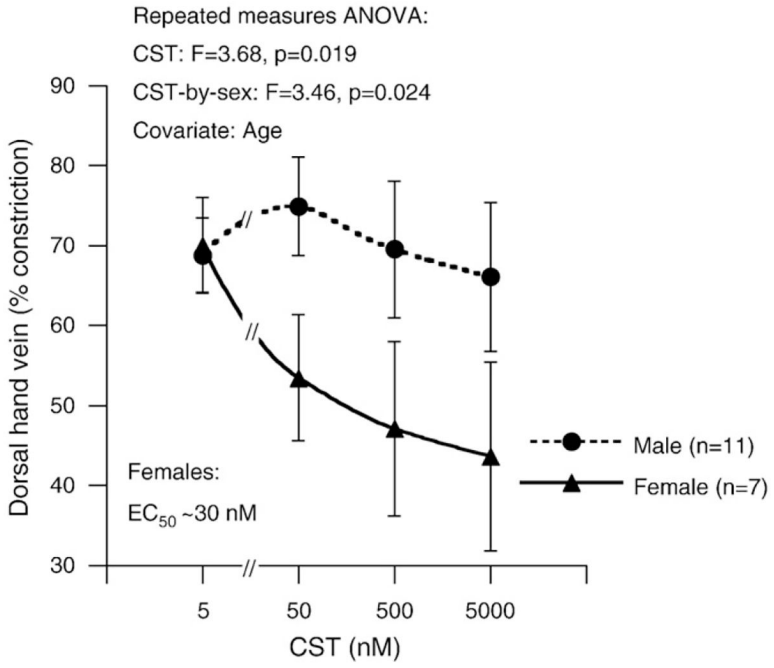

Fig. 7.

Vasodilation by exogenous CST infusion into the human dorsal hand vein: stratification by sex. CST exhibited dose-dependent vasodilation (p=0.019) in phenylephrine-induced venoconstriction (~70%), with the effect most prominent in female subjects (p=0.024; covariate: age). The F value (>1) indicates that the means are significantly different from one another. Assuming maximal venodilation with the highest concentration of CST (~5000 nM), the EC_{50} (semi-maximal effective concentration) for females was ~30 nM.

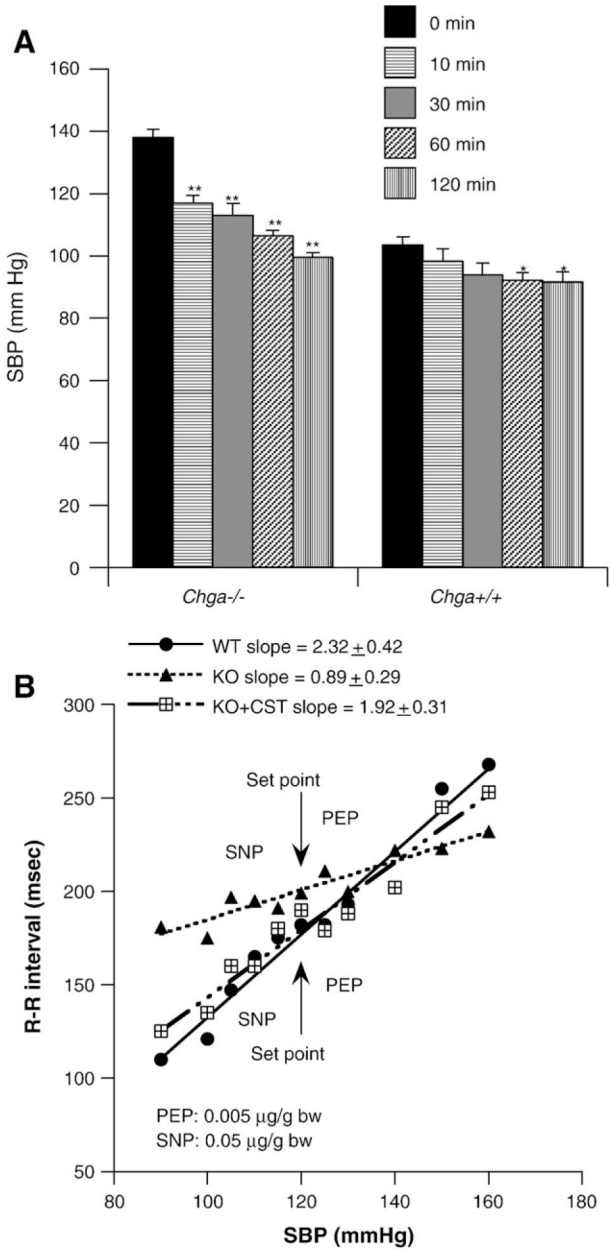

Fig. 8.

CST reversal of heightened SBP and dampened baroreflex sensitivity in *Chga*-KO mice. (A). Rescue from elevated SBP by exogenous CST: exaggerated SBP fall in *Chga*-KO mice. SBP was monitored by telemetry before and after administration of CST (2.5 mg/kg body weight, IP) at time 0 in wild-type (WT; n=4) and *Chga*-KO (n=4) mice. Results were analyzed by 2-way, repeated-measures ANOVA, evaluating the effects of time (p<0.001), mouse strain (p=0.009), or strain/peptide interaction (p<0.001). (B). Baroreceptor slope after treatment with phenylephrine (PE: 0.005 µg/g bw IV) or sodium nitroprusside (SNP: 0.05 µg/g bw IV) in unconscious WT and *Chga*-KO mice, or after supplementation of CST (4 µg/g bw IV) in KO mice. Slopes in line drawings are presented from one representative animal per group. The slope values presented at the top of the figure are the mean values ± one SEM

(ms/mmHg; n=8 animals/group). “Set point” refers to the initial/resting/starting point for each animal (for SBP, in mmHg, and R–R interval, in ms), prior to administration of drugs.

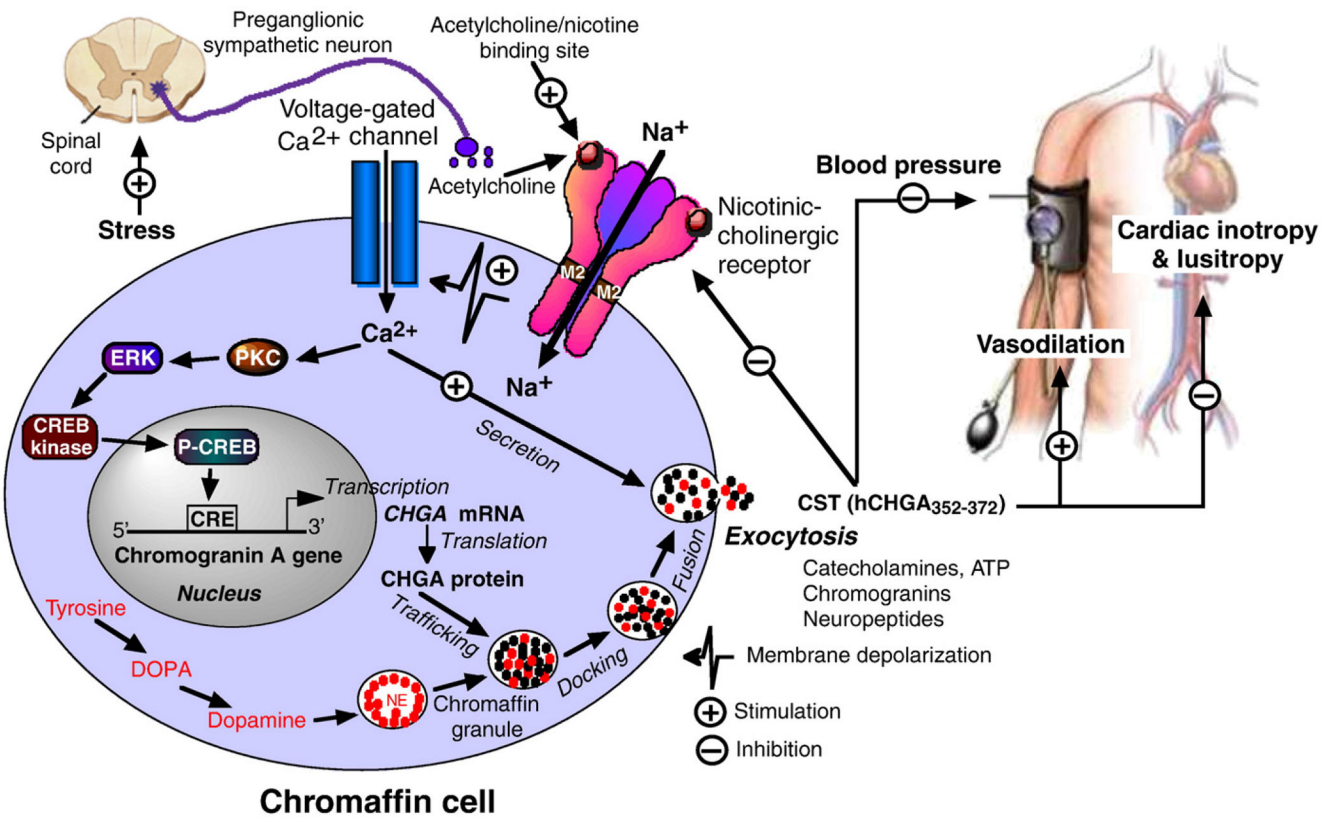

Fig. 9.

Model showing the autocrine–paracrine homeostatic regulation (negative-feedback) of catecholamine secretion by CST and its regulation of cardiovascular parameters including BP, cardiac contractility and vasodilation. Binding of nicotine (acetylcholine surrogate) to the nicotinic-cholinergic receptor induces extracellular Na^+ influx resulting in depolarization of the cell membrane that causes influx of calcium through voltage-gated calcium channels. Influx of calcium induces both catecholamine release by exocytosis (all- or-none secretion) and *Chga* gene transcription through a pathway involving activation of protein kinase C (*PKC*) and mitogen activated protein kinase (*MAPK*). CST formed in and secreted from chromaffin granules inhibits subsequent catecholamine secretion, decreases BP and cardiac contractility, and induces vasodilation. *NE*, norepinephrine; *ERK*, extracellular signal-regulated kinase; *CRE*, cAMP-response element; *CREB*, cAMP-response element-binding protein; and p-CREB: phosphorylated CREB.

Table 1

Inter-species homologies in the CST sequence in humans and other mammals including sequence variants in human CST (hCHGA$_{352\text{-}372}$). Amino acids at positions variant in human CST are shown in **bold** type. CHGA: chromogranin A. The typical dibasic proteolytic cleavage site at the carboxy-terminal side of CST is given in brackets, [RR]. For human CHGA, this [RR] site is $R^{373}R^{374}$.

Species	Amino acid sequence	Variant (frequency)	CST region in CHGA
Mouse	RSMRLSFRTRGY**G**FRDPG**L**QL[R**R**]	-	CHGA$_{364–384}$
Rat	RSMRLSFRARGY**G**FRDPG**L**QL[R**R**]	-	CHGA$_{367–387}$
Cow	RSMRLSFRARGY**G**FRGPG**L**QL[R**R**]	-	CHGA$_{344–364}$
Pig	RSMRLSFRAPAY**G**FRGPG**L**QL[R**R**]	-	CHGA$_{343–363}$
Horse	RSMKLSFRARAY**G**FRGPG**L**QL[R**R**]	-	CHGA$_{343–363}$
Chimp	SSMKLSFRARAY**G**FRGPG**P**QL[R**R**]	-	CHGA$_{354–374}$
Human			
Wild-type	SSMKLSFRARAY**G**FRGPG**P**QL[R**R**]	-	CHGA$_{352–372}$
Variant	SSMKLSFRARAY**S**FRGPG**P**QL[R**R**]	**G364S (3.1%)**	
Variant	SSMKLSFRARAY**G**FRGPG**L**QL[R**R**]	**P370L (0.6%)**	
Variant	SSMKLSFRARAY**G**FRGPG**P**QL[R**Q**]	**R374Q (0.3%)**	