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The catecholamine release-inhibitory peptide catestatin (chromogranin $A_{344-364}$) modulates myocardial function in fish

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SUMMARY

Catestatin (CST), the 21-amino acid, cationic and hydrophobic peptide proteolytically derived from the ubiquitous chromogranin A (CgA), is an endogenous inhibitor of catecholamine release, a potent vasodilator *in vivo* and an anti-hypertensive agent in mammals, including humans. Recently, we discovered that CST also functions as an important negative modulator of heart performance in frog and rat. To gain an evolutionary perspective on CST cardiotropism in fish, we analysed the influence of bovine CST ($CgA_{344-364}$) on the eel heart, as well as the eventual species-specific mechanisms of its myocardial action. Experiments were carried out on fresh-water eels (*Anguilla anguilla* L.) using an electrically paced isolated working heart preparation. Stroke volume and stroke work were used as measures of ventricular performance. Under basal conditions, CST (from 11 nmol l^{-1} to 165 nmol l^{-1}) caused a concentration-dependent negative inotropism, which was abolished by inhibitors of either β_1/β_2 (propranolol) or β_3 (SR_{59230}) adrenergic receptors, or by $G_{i/o}$ protein (PTx) or nitric oxide synthase (L-NMMA), or guanylate cyclase (ODQ) blockers. This suggests a β-adrenergic receptor-$G_{i/o}$ protein-NO-cGMP-dependent mechanism. By contrast, the CST-induced cardio-suppression was not influenced by atropine, unspecific muscarinic antagonist, thus excluding cholinergic receptor involvement. CST also counteracted the adrenergic (isoproterenol)-mediated positive inotropism. Under increased preload (i.e. Frank–Starling response) conditions, CST induced a significant increase of the Frank–Starling response, which was blocked by L-NMMA and thapsigargin, but independent from guanylate cyclase. In conclusion, this is the first report in fish that CST modulates myocardial performance under basal, as well as under increased preload, conditions and counteracts the adrenergic-mediated positive inotropism, which strikingly supports the evolutionary significance and establishes the cardioactive role of this peptide.

Key words: chromogranin A, inotropic agents, stroke volume, myocardial relaxation, nitric oxide synthase, SERCA2a, *Anguilla Anguilla* L.

INTRODUCTION

Chromogranin A (CgA), a 48 kDa acidic secretory protein, is the major member of the chromogranin/secretogranin family of glycoproteins expressed in all neuroendocrine cells (Winkler and Fisher-Colbrie, 1992). As first shown in the adrenal medulla, it is co-stored and co-released with catecholamines (CAs) from the secretory vesicles of the chromaffin cells (Takiyyuddin et al., 1990; Helle et al., 2007). CgA has also been detected in secretory granules of the heart. In the rat, it was found in atrial and ventricular Purkinje fibres containing the calcium channel alpha 1E subunit (Weiergraber et al., 2000), and in non-adrenergic myoendocrine atrial cells, in which it co-stores with atrial natriuretic peptides (Steiner et al., 1990). In humans, it colocalizes with brain natriuretic peptide (BNP) in ventricular myocytes of the dilated and hypertrophic heart (Pieroni et al., 2007). In addition to its crucial role in secretory vesicle biogenesis (Courel et al., 2006), CgA acts as a prohormone that generates several biologically active peptides, including the dysglycemic hormone pancreastatin (Tatemoto et al., 1986), the vasodilator vasostatin 1 (VS-1) (Aardal et al., 1993), the antimicrobial agent chromacin (Strub et al., 1996) and the catecholamine release-inhibitory peptide catestatin (Mahata et al., 1997; Mahata et al., 2003; Mahata et al., 2004).

Catestatin (CST; human $CgA_{352-372}$, bovine $CgA_{344-364}$) is a COOH-terminal CgA fragment (Fig. 1). It is the first known endogenous compound able to inhibit *in vitro* catecholamine release from both chromaffin cells and noradrenergic neurons by acting as a non-competitive nicotinic cholinergic antagonist (Mahata et al., 1997). CST is a potent vasodilator *in vivo* in rat (Kennedy et al., 1998) and human (Fung et al., 2010), and is an anti-hypertensive agent (Mahapatra et al., 2005). In fact, low levels of CST are associated with augmented adrenergic responses to stressors and an increased risk of hypertension (O'Connor et al., 2002). Recently, in the isolated working frog heart, CST was found to function also as an important cardio-inhibitor that is able to reduce stroke volume (V_S) and stoke work (W_S) and to counteract the positive inotropism exerted by both β-adrenergic (isoproterenol, ISO) and endothelin-1 stimulation (Mazza et al., 2008). Similarly, in the isolated and perfused Langendorff rat heart, the peptide induces negative inotropism and lusitropism, inhibiting ISO- and endothelin-1-induced positive inotropism and coronary constriction (Angelone et al., 2008). Thus, besides its anti-hypertensive properties, CST is now emerging as a novel cardiac modulator, which would protect the heart against excessive systemic and/or intra-cardiac excitatory stimuli (e.g. catecholamines and endothelin-1).

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                                                                      10                  20
Homo sapiens (human CHGA352-372):                             S S M K L S F R A R A Y G F R G P G P Q L
Pan troglodytes (chimpanzee CHGA354-374):                     S S M K L S F R A R A Y G F R G P G P Q L    (100%)
Macaca fascicularis (macaque CHGA353-373):                    R S M K L S F R A R A Y G F R G P G P Q L    (95%)
Macaca mulatta (rhesus monkey CHGA350-370):                   R S M K L S F R A R A Y G F R G P G P Q L    (95%)
Equus caballus (horse CHGA343-363):                           R S M K L S F R A R A Y G F R G P G L Q L    (90%)
Ratus norvegicus (rat CHGA367-387):                           R S M K L S F R A R A Y G F R D P G P Q L    (90%)
Mus musculus (mouse CHGA364-384):                             R S M K L S F R T R A Y G F R D P G P Q L    (86%)
Sus scrofa (pig CHGA343-363):                                 R S M K L S F R A P A Y G F R G P G L Q L    (86%)
Bos taurus (cattle CHGA344-364):                              R S M R L S F R A R G Y G F R G P G L Q L    (90%)
Gallus gallus (jungle fowl CHGA365-385):                      R S M K M A F R S H K Y D F S S P E E D V    (38%)
Rana ridibunda (marsh frog CHGA300-320):                      R S M K I P T K D Q K Y E P A S E E H E D    (19%)
Xenopus tropicalis (African clawed frog CHGA335-355):         Q S M K I P P T E Q K Y N L G G P E P D D    (33%)
Xenopus laevis (western clawed frog CHGA335-355):             Q S M K I P P M K M K Y N L G G P E P E D    (33%)
Danio rerio (zebrafish CHGA266-286):                          V D E R W E F K - H S K E R E D P E G D L W  (19%)
Consensus CST sequence in vertebrates:                        R S M K L S F R A R A Y G F R G P G P Q L W
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Fig. 1. Homology of the catestatin (CST) sequence in vertebrate species. The alignment of the CST domain in vertebrate species was performed using ClustalW Alignment (MacVector version 9.0) and the percentages of homology were calculated. The most conserved amino acids in the CST domain of vertebrate species are highlighted in grey. The percentages of homology amongst the amino acid sequences of vertebrate CST are shown in parenthesis. The following NCBI RefSeq Accession numbers were used for CST sequences in different species: human (NM_001275), chimpanzee (XM_510135), rhesus monkey (XM_001092629), horse (NM_001081814), rat (NM_021655), mouse (NM_007693), pig (NM_001164005), cattle (NM_181005), jungle fowl (XM_421330), African clawed frog (NM_001094724) and zebrafish (NM_001006059). GenBank Accession numbers for CST sequences in macaque, Western clawed frog and marsh frog were AB169793, BC080353 and AF139924, respectively.

The heart of many teleosts, including the eel, is exposed to the stimulatory effects of circulating and intracardiac catecholamines, particularly under stress conditions (see Imbrogno et al., 2003), which might become harmful in the absence of local counter-regulatory mechanisms. In fact, fish exposed to stress do not survive because of a sustained sympathetic activity and strenuous escaping behavior (van Raaij et al., 1996; Schjolden et al., 2005).

We previously reported that in the eel several humoral and neuro-endocrine factors exert a cardio-inhibitory protection (Imbrogno et al., 2003; Imbrogno et al., 2004; Imbrogno et al., 2006). Among others, the NH_2-terminal fragment of CgA, VS-1, has been shown to exert cardio-suppressive inotropic influences under both basal and beta-adrenergic-stimulated conditions (Imbrogno et al., 2004). Accordingly, we hypothesize that, as in frog and rat, CST in the eel heart is part of a counter-regulatory network that protects the heart from excessive excitatory stimuli, such as those deriving from activation of the adrenergic system.

To test this hypothesis, we analysed the influence of bovine CST ($CgA_{344-364}$) on the isolated working eel (*Anguilla anguilla*) heart to explore its cardiotropic role in fish and the eventual species-specific mechanisms underlying its myocardial action. Bovine CST has been thoroughly characterized in mammals (Mahata et al., 1997; Mahata et al., 2000; Mahata et al., 2004), and we have already established that this peptide is effective in non-mammalian vertebrates (Mazza et al., 2008). Although 19% homology exists between human and zebrafish CST, and 24% homology between bovine and zebrafish CST, piscine CST has not yet been characterized. We demonstrate, for the first time in fish, that CST exerts a direct suppressive action on basal heart performance, and is also able to counteract the beta-adrenergic-mediated positive inotropism. When tested on Starling-treated preparations, CST significantly increased the Starling (length-tension or heterometric) response. However, whereas under basal (unstimulated) conditions the CST effect involved the adrenergic system and occurred *via* a NO-cGMP cascade, in Starling-treated hearts it involved NO synthase (NOS) and SR-CA^{2+}ATPase (SERCA2a) pumps, but not guanylyl cyclase (GC).

These data might contribute to the tracking of the evolutionary 'ancestral' function of CgA-derived peptides. At the same time, the comparative approach using different cardiac morpho-dynamic designs – teleost heart (present work), amphibian heart (Mazza et al., 2008), mammalian heart (Angelone et al., 2008) – might help to reveal mechanistic aspects of CST action that could be of interest in mammalian and human cardio-protection against stress.

MATERIALS AND METHODS

Animals

We used specimens of freshwater European eel (*Anguilla anguilla* L.), weighing 78.5±2.3 g (mean ± s.e.m., N=50). Fish were provided by a local hatchery and kept at room temperature (18–20°C) for 5-7 days. Each eel was anaesthetized with tricaine methane sulfonate (Sigma Chemical Co., St Louis, MO, USA). In accordance with the accepted standards of animal care, the experiments were organized to minimize stress and number of animals used.

Isolated and perfused working heart preparations

The hearts, isolated and connected to a perfusion apparatus as previously described (Imbrogno et al., 2001), received Ringer's solution from an input reservoir and pumped against an afterload pressure given by the height of an output reservoir. The composition of the perfusate (in mmol l^{-1}) was: NaCl 115.17, KCl 2.03, KH_2PO_4 0.37, $MgSO_4$ 2.92, $(NH_4)_2SO_4$ 50, $CaCl_2$ 1.27, glucose 5.55, Na_2HPO_4 1.90; pH was adjusted to 7.7–7.9 by adding $NaHCO_3$ (about 1 g l^{-1}) (Imbrogno et al., 2001); the Ringer's solution was equilibrated with a mixture of O_2:CO_2 at 99.5:0.5%. Experiments were carried out at room temperature (18–20°C). Hearts were stimulated with an LE 12006 stimulator (frequency identical to that of control, non-paced hearts; pulse width fixed at 0.1 ms; voltage: 1.2±0.1 V, means ± s.e.m.).

Measurements and calculations

Pressure was measured through T-tubes placed immediately before the input cannula and after the output cannula, and connected to two MP-20D pressure transducers (Micron Instruments, Simi Valley, CA, USA) in conjunction with a Unirecord 7050 (Ugo Basile, Comerio, Italy). Pressure measurements (input and output) were expressed in kilopascal (kPa) and corrected for resistances of cannula and of tube length. Heart rate (f_H) was calculated from pressure recording curves. Cardiac output ($\dot{Q}$) was collected over 1 min and weighed; values were corrected for fluid density and expressed as volume measurements. The afterload (mean aortic pressure) was calculated

as two-thirds diastolic pressure plus one-third maximum pressure. Stroke volume (V_S; ml kg^{-1}; $\dot{Q}/f_H$) was used as a measure of ventricular performance; changes in V_S were considered to be inotropic effects. $\dot{Q}$ and V_S were normalized per kilogram of wet body mass. Ventricular stroke work [W_S; mJ g^{-1}; (afterload-preload)×V_S/ventricle mass] served as an index of systolic functionality.

Experimental protocols

Basal conditions

Isolated perfused hearts were allowed to maintain a spontaneous rhythm for up to 15–20 min. In all experiments, the control conditions were established at a mean output pressure of about 3 kPa, with a $\dot{Q}$ set to 10 ml min^{-1} kg^{-1} body mass by appropriately adjusting the preload (filling pressure). These values are within the physiological range [for references, see Imbrogno et al. (Imbrogno et al., 2001)]. Cardiac parameters were simultaneously measured during experiments. To analyse the inotropic effects distinct from the chronotropic actions of substances, the preparations were electrically paced. Hearts that did not stabilize within 15–20 min from the onset of perfusion were discarded.

Drug application

After the 15–20 min control period, paced hearts were perfused for 20 min with non-re-circulating Ringer's solution enriched with CST at increasing concentrations to construct cumulative concentration–response curves.

Heart preparations were used to test the effects of 110 nmol l^{-1} of CST in the presence of adrenergic antagonists (phentolamine, propranolol and SR_{59230}), a cholinergic antagonist (atropine), the nitric oxide synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA) and the soluble guanylate cyclase (GC) specific inhibitor [1H-(1,2,4)oxadiazole-(4,3-a)quinoxalin-1-one (ODQ)]. In the above-mentioned protocols the hearts were perfused for 20 min with Ringer's solution enriched with the specific drug before the addition of CST. To analyse the effects of CST on the isoproterenol (ISO) response, CST-stimulated hearts were perfused with Ringer's solution containing CST (110 nmol l^{-1}) plus ISO (100 nmol l^{-1}).

In another set of experiments the effects of CST (110 nmol l^{-1}) were tested after inhibition of G-proteins by the *Bordetella pertussis toxin* (PTx), commonly used to identify G protein-regulated signalling pathways coupled to $G_{i/o}$ subunits. In this case the hearts were pre-incubated for 60 min with PTx. PTx catalyses ADP-ribosylation of the $G_{i/o}$ α-subunit, uncoupling G_i membrane receptor interaction [see Ai et al. (Ai et al., 1998), and references therein].

Cardiac parameters were measured at the end of the perfusion period with the specific substance constantly administered in the Ringer's solution.

Frank–Starling response

To assess the interaction between CST and the Frank–Starling response, a Starling curve was generated by varying the atrial reservoir height to alter the preload on the *in vitro* heart. After baseline assessment, the atrial reservoir height was returned to basal conditions and a second Starling curve was generated in the presence of CST.

A second series of experiments was performed to investigate the CST-dependent transduction pathway. A first Frank–Starling curve was generated in the presence of CST; then, the input pressure was returned to the control condition and a second curve was generated in the presence of CST plus the NOS inhibitor L-NMMA, or the soluble gunylate cyclase blocker ODQ, or the β_3-AR specific antagonist SR_{59230}, or after the inhibition of SERCA2a pumps by thapsigargin.

The putative influence of the first Starling curve (baseline assessment) on the second Starling curve (treated hearts) was excluded according to our previous studies (Imbrogno et al., 2003; Imbrogno et al., 2001).

Phospho-eNOS detection

To assess whether the CST treatment induced eNOS phosphorylation, we analysed the e-NOS/phospho-eNOS ratio in both Starling-treated hearts and Starling-treated hearts in the presence of CST. Eel ventricle samples (*N*=3 for each condition: Starling response, Starling response plus CST) were rapidly immersed in liquid nitrogen and stored at –80°C. Ventricles were prepared according to Amelio et al. (Amelio et al., 2006). Proteins were separated on 8% SDS–PAGE gels, transferred to membrane, blocked with non-fat dried milk and incubated overnight at 4°C with polyclonal rabbit anti-eNOS and phospho-eNOS antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:1000 in TBS-Tween containing 5% bovine serum albumin (BSA). The anti-rabbit peroxidase-linked secondary antibody (Santa Cruz Biotechnology) was diluted 1:2000 in TBS-Tween containing 5% non-fat dried milk.

Immunodetection and densitometric analysis

Immunodetection was performed using an enhanced chemiluminescence kit (ECL PLUS, GE Healthcare Europe, Milan, Italy). Autoradiographs were obtained by exposure to X-ray films (Hyperfilm ECL, GE Healthcare Europe). Immunoblots were digitalized and densitometric analysis of the bands was carried out using NIH Image 1.6 for the Macintosh computer based on 256 grey values (0=white; 256=black).

Statistics

Percentage changes were evaluated as means ± s.e.m. of percentage changes obtained from individual experiments. Because each heart acted as its own control, the statistical significance of differences within each group was assessed using the paired Student's *t*-test (P<0.05). Comparisons between groups were made using two-way analysis of variance (ANOVA). Significant differences were detected using Duncan's multiple-range test (P<0.05).

The results of absorbance measurements and the grey values obtained from the densitometric analysis were expressed as means ± s.e.m. of determinations for each sample. The difference between groups was assessed by Student's *t*-test: statistical significance, *P<0.001.

Drugs and chemicals

Bovine CST ($bCgA_{344-364}$) peptide was synthesized by the solid-phase method, using 9H-(f)louren-9-yl(m)eth(o)xy(c)arbonyl protection chemistry, as previously described (Mahata et al., 2000). Peptides were purified to >95% homogeneity by preparative reverse-phase HPLC (RP-HPLC) on C-18 silica columns. Authenticity and purity of peptides were further verified by analytical chromatography (RP-HPLC), and electrospray-ionization or matrix-assisted laser desorption/ionization mass spectrometry. N^G-monomethyl-L-arginine (L-NMMA), 1H-[1,2,4]oxadiazole-[4,3-a]quinoxalin-1-one (ODQ), ISO, propranolol, phentolamine, atropine, PTx and 3-(2-ethylphenoxy)-1-{[(1S)-1,2,3,4-tetrahydronapth-1-yl]amino}-(2S)-2-propanol oxalate salt (SR_{59230}) were purchased from the Sigma Chemical Company (St Louis, MO, USA). Thapsigargin was

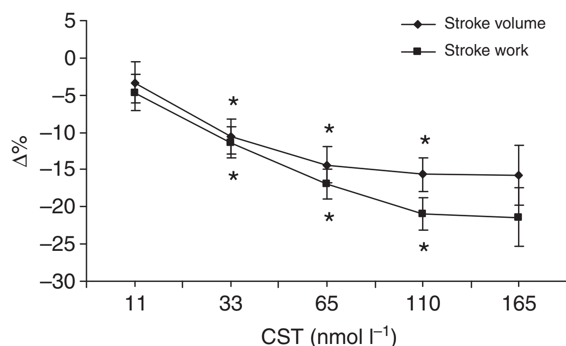

Fig. 2. Cumulative concentration–response curve for CST on stroke volume (V_S) and stroke work (W_S). Percentage changes were evaluated as means ± s.e.m. of four experiments. Significant difference from control values (*t*-test) is indicated by an asterisk (P<0.05).

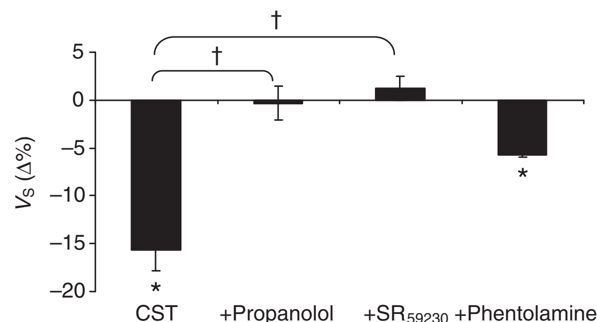

Fig. 3. Effects of CST (110 nmol l^{-1}) on V_S before and after treatment with propranolol (0.1 μmol l^{-1}), SR_{59230} (0.1 μmol l^{-1}) or phentolamine (0.1 μmol l^{-1}). Percentage changes were evaluated as means ± s.e.m. of three to four experiments for each drug. Significant difference from control values (*t*-test) is indicated by an asterisk (P<0.05); dagger indicates a significant difference between groups (ANOVA, Duncan's test; P<0.05).

purchased from Calbiochem (Milan, Italy) and was used in a darkened perfusion apparatus to prevent degradation.

All the solutions were prepared in double-distilled water (ODQ was prepared in ethanol); dilutions were made in Ringer's solution immediately before use.

RESULTS

Isolated heart preparation

After equilibration, the standardized *in vitro* isolated and perfused heart preparation worked at physiological loads and generated values of output pressure, f_H, $\dot{Q}$ and V_S that mimicked the *in vivo* physiological values of the animal (see Imbrogno et al., 2001).

The performance of the eel heart was stable for more than 2 h, after which the heart fell into a hypodynamic state characterized by a linear decrease in cardiac output, without significant changes in heart rate (see Imbrogno et al., 2001).

Baseline variables for the resting heart preparations were: output pressure (kPa), 3.25±0.23; f_H (beats min^{-1}), 49.59±13.13; $\dot{Q}$ (ml min^{-1} kg^{-1}), 10.45±1.01; V_S (ml kg^{-1}), 0.23±0.06. Data are expressed as mean ± s.e.m.; N=50.

Effects of CST on basal mechanical performance

To study the effects of CST under basal (unstimulated) conditions, cumulative concentration–response curves of the peptide were generated. CST (concentrations ranging from 11 to 165 nmol l^{-1}) induced a negative inotropism, as shown by a significant decrease in V_S at and above 33 nmol l^{-1}, which reached a maximum (about 15% of reduction) at a concentration of 110 nmol l^{-1} (Fig. 2).

Cholinergic and adrenergic receptors

The analysis of the involvement of cholinergic and adrenergic receptors in the CST response revealed that unspecific muscarinic inhibition by atropine did not influence the CST-dependent inotropism, thus excluding the involvement of cholinergic receptors (data not shown). To analyse the role of adrenergic receptors, eel heart preparations were pretreated with either β_1/β_2-(propranolol), or β_3-(SR_{59230}) or α-(phentolamine) adrenergic antagonists prior exposure to CST. Whereas pretreatment with phentolamine only reduced CST-mediated inotropism, SR_{59230} and propranolol blocked the CST effects, thus uncovering a preferential involvement of the β-adrenergic receptor system in CST-induced cardiosuppression (Fig. 3). All of these treatments alone did not significantly modify basal cardiac performance (data not shown).

$G_{i/o}$ proteins and NO-cGMP signal transduction pathway

G-proteins process many biological signals in intracellular language. To examine whether they are involved in the negative inotropic action exerted by CST, we pretreated eel cardiac preparations with pertussis toxin (PTx; 0.01 nmol l^{-1}), which uncouples signal transduction between several families of receptors and $G_{i/o}$ proteins. Although PTx alone did not modify basal cardiac performance (data not shown), its pretreatment abolished the inotropic effect of CST (V_S values for PTx plus CST were 1.3±1.5%).

NO, *via* activation of GC, is an important modulator of cardiac performance. In the eel, its role in mediating inotropic effects induced by cardioactive agents has been extensively demonstrated (Imbrogno et al., 2003; Imbrogno et al., 2004; Imbrogno et al., 2006). To analyse whether the CST-induced cardiac response involves a NO-cGMP pathway, the heart preparations were pretreated with either L-NMMA (10 μM; a NOS inhibitor) or ODQ (10 μM; a guanylyl cyclase blocker). These treatments abolished the CST-induced basal inotropic effects, demonstrating their dependence on a NO-cGMP mechanism (Fig. 4).

Cardioinhibitory activity of CST on adrenergic-stimulated preparations

CST has been shown to counteract the ISO-mediated positive inotropism in frog (Mazza et al., 2008) and rat (Angelone et al., 2008) hearts. Exposure of the eel heart preparations to ISO alone (100 nmol l^{-1}) causes the classical positive inotropism in 70% of preparations and a negative inotropic effect in 30% of preparations (Imbrogno et al., 2006). The effect induced by ISO was studied after pretreating the hearts with CST. Results indicated an antagonistic effect of CST towards the ISO-dependent positive inotropism (V_S values for ISO alone were 13±1.5% and for ISO plus CST were 0.65±1.4%).

CST and the Frank–Starling response

Like in many fish, the eel heart is very sensitive to the Frank–Starling response, i.e. the heterometric regulation that contributes to increased cardiac output in association with increased filling pressure, as occurs during periods of exercise or of increased venous return (Farrell and Jones, 1992).

The influence of CST on the Frank–Starling response of the isolated and perfused eel heart was studied by increasing the preload in the presence and absence of CST (110 nmol l^{-1}). Two-way ANOVA analysis showed a significant increase of the

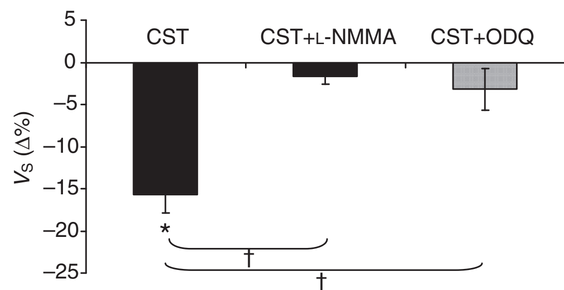

Fig. 4. Effects of CST (110 nmol l^{-1}) on V_S before and after treatment with N^G-monomethyl-L-arginine (L-NMMA; 10 μmol l^{-1}) or 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ; 10 μmol l^{-1}). Percentage changes were evaluated as means ± s.e.m. of three to four experiments for each drug. Significant difference from control values (*t*-test) is indicated by an asterisk (P<0.05); dagger indicates a significant difference between groups (ANOVA, Duncan's test; P<0.05).

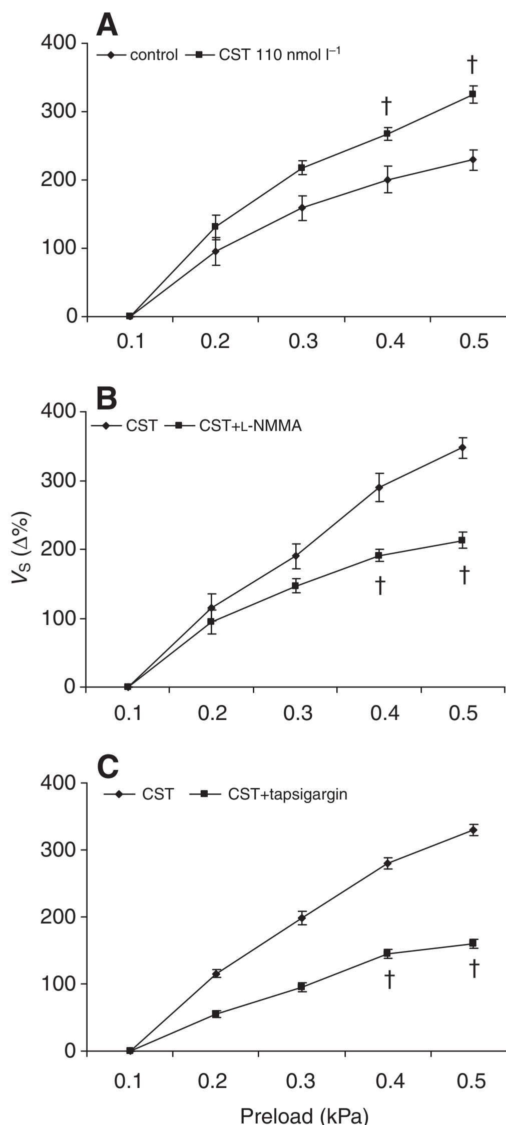

Fig. 5. Effects of preload elevation on V_S under control conditions and after treatment with CST (110 nmol l^{-1}; A), under treatment with CST and CST plus L-NMMA (10 μmol l^{-1}; B), and under treatment with CST and CST plus thapsigargin (0.1 μmol l^{-1}; C). Percentage changes were evaluated as mean ± s.e.m. (N=5 for each group). Comparison between groups was made using two-way ANOVA analysis ($^{\dagger}P$<0.05).

Frank–Starling curves in the presence of CST (Fig. 5), suggesting the existence of a CST-dependent modulation of the cardiac heterometric mechanism in the eel.

Frank–Starling response and CST signal transduction

In this study, we have documented that the CST-induced basal negative inotropism in the eel heart involves β_3-AR. To verify whether β_3-AR also mediates the effect of CST on the Frank–Starling response, evaluation was carried out in presence and absence of a specific β_3-AR inhibitor, SR_{59230} (100 nmol l^{-1}). Of note, the results obtained excluded β_3-AR activation (data not shown), suggesting that the mechanism underlying the CST-induced basal negative inotropism differs from the mechanism that underlies the CST modulation of the Frank–Starling response.

We previously reported that intracardiac NO increases the sensitivity of the *in vitro* eel heart to filling pressure changes (Imbrogno et al., 2001). This, independently from the guanylate cyclase/cGMP pathway, occurs through a modulation of the SERCA2a pumps (Garofalo et al., 2009). To evaluate the involvement of NOS, GC and SERCA pumps in the CST-induced increase of the Frank–Starling response, eel heart preparations were pretreated with L-NMMA, ODQ or thapsigargin, respectively. Results showed that, although L-NMMA and thapsigargin (Fig. 5) abolished the effect of CST on the Frank–Starling response, ODQ did not modify it (data not shown), thereby excluding cGMP-dependent signaling in the CST modulation of the Frank–Starling response. Under basal conditions, thapsigargin (10^{-8}–10^{-5} mol l^{-1}) did not modify basal mechanical performance (Garofalo et al., 2009).

Phospho-eNOS expression

Western blotting analysis showed the presence of phopsho-eNOS (p-eNOS) in the hearts of *A. anguilla* either after the Frank–Starling response or after the Frank–Starling response performed in the presence of CST (110 nmol l^{-1}). An immunoreactive band of approximatively 140 kDa, corresponding to the known p-eNOS molecular weight, was detected. Densitometric quantification of the blots revealed that hearts exposed to Frank–Starling curves performed after CST treatment have a higher p-eNOS expression (27.41%; P<0.001) than the control (Fig. 6).

DISCUSSION

CST is the most potent endogenous non-competitive antagonist of nicotine-evoked catecholamine release from chromaffin cells and noradrenergic neurons (Mahata et al., 1997). It also acts as a potent vasodilator *in vivo* in rat (Kennedy et al., 1998) and in human (Fung et al., 2010), and an anti-hypertensive agent (Mahapatra et al., 2005). Recent work from our lab revealed that, in addition to its role in blood pressure control, CST directly suppresses the mechanical performance of both non-stimulated and adrenergically stimulated cardiac preparations from frog (Mazza et al., 2008) and rat (Angelone et al., 2008).

In the present study, we show for the first time in a fish heart that CST modulates myocardial function of the eel *A. Anguilla*, both under basal and increased preload conditions, being also able to counteract β-adrenergic-mediated positive inotropism.

Effects of CST on basal mechanical performance

Our data indicate that in the eel CST stimulation depresses cardiac contractility, which supports a ubiquitous cardio-depressive action of

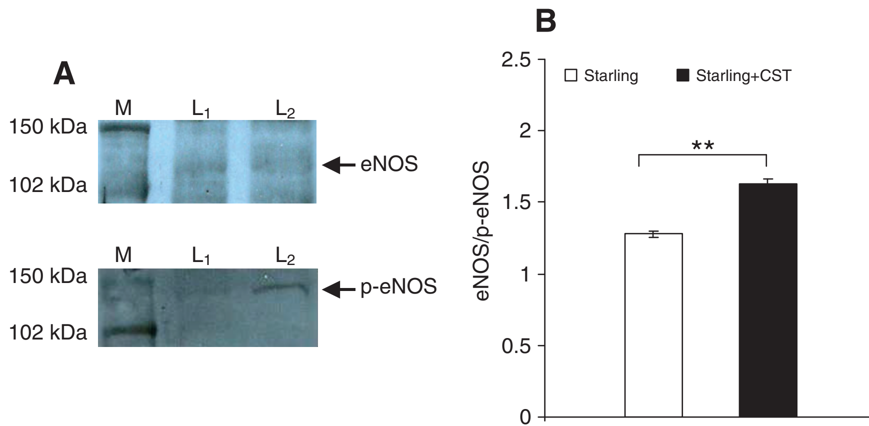

Fig. 6. (A) Western blotting of total endothelial nitric oxide synthase (eNOS) and phosho-eNOS (p-eNOS) in heart extracts (M, Marker; L_1, Starling; L_2, Starling in the presence of CST). (B) Densitometric quantification of p-eNOS and total eNOS. Data are means ± s.e.m. of four determinations from each animal (*N*=6). Statistical differences were evaluated by Student's *t*-test (***P*<0.001).

this peptide in vertebrates. The resulting CST-induced inotropism was comparable to that elicited on the eel heart by human recombinant VS-1 (Imbrogno et al., 2004). Of note, CST and VS-1, i.e. the COOH- and the NH_2-terminal domains of CgA, respectively, elicit cardio-inhibitory effects of the same order of magnitude at low concentrations that are close to the physiological human plasma levels of CgA (from 0.5 up to 2 or 3 $nmol\,l^{-1}$) (Ceconi et al., 2002) and CST (1.3 to 4.4 $nmol\,l^{-1}$) (O'Connor et al., 2002; Fung et al., 2010). The biological significance of such apparently redundant molecular strategy remains to be clarified. However, conceivably, processing more than one cardioactive peptide from the same prohormone might be advantageous in maintaining homeostasis. For example, a stimulus-proteolytic-secretion coupling of CgA might achieve selective intracardiac actions in a spatiotemporal and tissue-specific manner. A similar cardiovascular case is represented by the vasodilatory, diuretic and natriuretic properties of the peptide hormones (e.g. atrial natriuretic peptide and vessel dilator) derived from the pro-atrial natriuretic peptide (Vesely, 2006). Numerous monobasic and dibasic amino acid residues in CgA indicate potential sites for endoproteolytic processing by the prohormone convertases (subtilisin-like and trypsin-like) PC1/3 and PC2 that occur as co-stored components of neurosecretory granules (Seidah and Chretien, 1999), including the myocardial-specific granules (Muth et al., 2004). Four N-terminal CgA-derived peptides (CgA4-113, CgA1-124, CgA1-135 and CgA1-199) have been characterized from rat heart homogenates, which is consistent with cleavage into betagranins, containing the homologous VS-1 motif (Glattard et al., 2006). Thus, the possibility exists that in the eel heart CST, locally generated from intracardiac CgA, participates in the autocrine-paracrine modulation of myocardial performance under both unstimulated and adrenergically stimulated (see below) conditions.

Signal transduction mechanisms

Surface receptors for CST-dependent cardiovascular effects have not yet been documented; therefore, whether CST acts on the heart *via* classic receptor-ligand interactions remains to be elucidated. CST inhibits catecholamine release from sympathochromaffin cells by targeting nicotinic acetylcoline receptors *in vivo* in mice (Mahata et al., 2003), and *in vitro* in rat PC12 cells (Mahata et al., 2003; Mahata et al., 2004) and in primary bovine chromaffin cells (Mahata et al., 1997). So far, the involvement of muscarinic receptors in the CST-dependent inotropic and lusitropic effects has been excluded in rat (Angelone et al., 2008). Apart from eventual specific receptor interactions (including those with cardiac nicotinic receptors), the finding in the eel heart that pretreatment with the non-specific muscarinic antagonist atropine did not affect the CST response points to a muscarinic receptor-independent effect. Moreover, whereas pretreatment with the α-adrenergic inhibitor phentolamine only reduced CST-mediated inotropism, SR_{59230} (β_3) and propranolol (β_1/β_2) adrenergic antagonists blocked the peptide effects, thus indicating a preferential involvement of the β-adrenergic receptor system in CST signalling. In agreement with the eel data, in the rat heart the CST-induced negative inotropism is abolished by $\beta_{1/2}$-AR

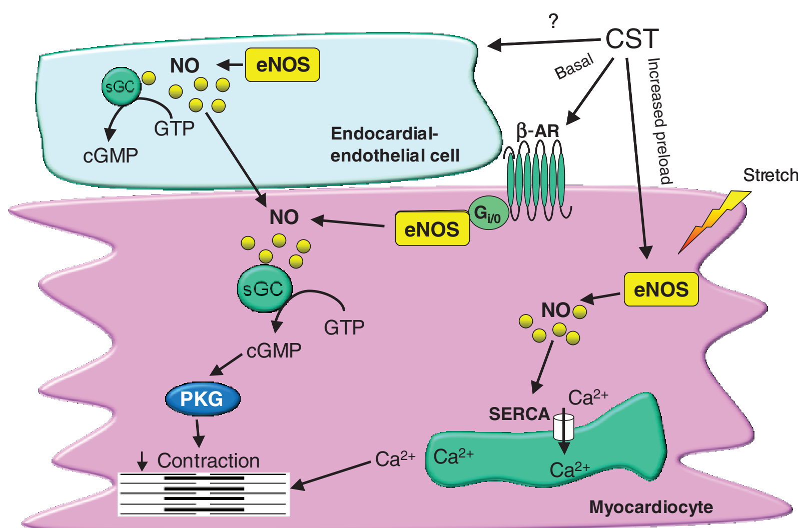

Fig. 7. Schematic diagram of putative CST signaling in myocardial cells and its modulatory action on the mechanical performance of the *A. anguilla* heart. Under basal conditions, the cardio-inhibitory action of CST involves β-ARs, $G_{i/o}$ proteins and the NO-cGMP pathway. Under increased preload conditions, CST enhances the heart sensitivity to the Frank–Starling response through an NO-dependent regulation of SR Ca^{2+} reuptake. β-AR, β-adrenergic receptor; eNOS, endothelial NOS; PKG, protein kinase G; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPases; sGC, soluble guanylate cyclase; cGMP, cyclic guanosine monophosphate; GTP, guanosine triphosphate; NO, nitric oxide; $G_{i/0}$, $G_{i/0}$ proteins.

inhibition by nadolol, is reduced by α-AR antagonism by phentolamine, and is unaffected by atropine-induced cholinergic receptor inhibition (Angelone et al., 2008). Although the specific implication of the adrenergic receptors in the CST-induced effects remains to be clarified, our data reveal aspects of unity in the cardiac action of CST between the two vertebrates. Similarly, in the eel, CST-dependent effects are associated with a $G_{i/o}$-dependent transduction mechanism, as they are abolished by PTx pretreatment. A sensitivity to PTx, already observed for CST-evoked histamine release in rat mast cells (Krüger et al., 2003), has been demonstrated for CST-dependent inotropic and lusitropic effects in the rat heart (Angelone et al., 2008). However, it is unknown whether the involvement of β-ARs and $G_{i/o}$ proteins in the CST-induced inotropic effects observed in the eel occurs through a direct receptor interaction and/or through a perturbation of spatially colocalized receptor systems in cell membrane microdomains (e.g. caveolae) (Tota et al., 2007; Dondossola et al., 2010).

In the working eel heart, intracardiac NO acts *via* sGC signalling to elicit an important modulation of basal and chemically stimulated mechanical performance (see Imbrogno et al., 2001; Imbrogno et al., 2003; Imbrogno et al., 2004; Imbrogno et al., 2006). In the present work, we report that the NO-cGMP pathway is involved in the negative inotropism induced by CST (Fig. 7), as it is abolished by pretreatment with either the NOS inhibitor L-NMMA or the sGC blocker ODQ. This agrees with the involvement of this signaling pathway in the CST-dependent cardiosuppression observed in frog and rat (Angelone et al., 2008; Mazza et al., 2008), and with the NO-cGMP-dependent negative inotropism induced by VS-1 in both eel and rat (Imbrogno et al., 2004; Cappello et al., 2007). Although detailed molecular mechanisms are not yet completely established, the finding that the cardiotropic action of CST in at least three classes of vertebrates involves converging transduction cascades, underlines the key role of some mediators (i.e. NO) in the network of CST-elicited transduction pathways.

Cardioinhibitory activity of CST on ISO-stimulated preparations

In the eel heart, ISO, a non-specific β-AR agonist, produces a classic positive inotropic response. However, in the 30% of preparations, a negative inotropic effect involving β_3-AR has been observed (Imbrogno et al., 2006). Studies in frog and rat have proposed CST as a homeostatic regulator of the cardiovascular system that is able to counteract the excitatory adrenergic influences (Angelone et al., 2008; Mazza et al., 2008). Our results extended this CST-dependent counteraction to a fish heart, as the positive inotropic effect induced by ISO in the eel was blocked by CST pretreatment. Notably, analogous anti-adrenergic actions have been described for NH_2-terminal CgA peptides in eel (VS-1) (Imbrogno et al., 2004), frog (VS-1, CgA7-57, CgA1-40, CgA47-66) (Corti et al., 2004; Tota et al., 2003) and rat (VS-1, CgA1-64) (Cerra et al., 2006; Cerra et al., 2008).

Thus, we hypothesize that CST, as well as other CgA fragments, acts as a natural protective factor against excessive stimuli, particularly those induced by sustained activation of the adrenergic system.

Frank–Starling response

Heterometric regulation (the Frank–Starling mechanism) is an intrinsic property of all vertebrate hearts. Fish differ from other vertebrate classes in that they modulate cardiac output *via* changes in stroke volume rather than in heart rate (Farrel and Jones, 1992; Tota and Gattuso, 1996; Olson, 1998).

CST increases the sensitivity of the eel heart to the Starling response. In fact, ANOVA testing revealed significant differences between untreated and CST-treated hearts. It is noteworthy that the CST influence appears to be similar to that exerted by NO, as recently documented by us in the *A. anguilla* heart (Garofalo et al., 2009). This suggests that in this teleost CST-dependent Frank–Starling modulation can be ascribed to NO generation. This was evidenced by the significant reduction of the Starling response that was observed after inhibition of NOS by L-NMMA, and was further supported by an increased p-eNOS expression in CST-treated hearts. However, pretreatment with a GC inhibitor, ODQ, did not influence the effects of CST on the Frank–Starling response, suggesting an action mechanism that is dependent on NO but independent of its classical cGMP signaling. We recently reported in the same heart preparation that NO, without activation of cGMP-dependent pathways, directly modulates the Frank–Starling response through a beat-to-beat regulation of calcium reuptake by SERCA2a (Garofalo et al., 2009). The present results support the hypothesis that NO directly influences SERCA2a activity in the eel heart. In fact, we observed that the CST-induced increase of the Frank–Starling response was significantly reduced by thapsigargin-dependent inhibition of SERCA2a pumps. This allows us to suggest that CST activates the release of NO, which, in turn, modulates the response to preload increases *via* a direct regulation of SR Ca^{2+} reuptake (Fig. 7).

Conclusions

The present study demonstrates that CST exerts a remarkable modulatory action on the mechanical performance of the *A. anguilla* heart, under both basal and increased preload conditions. These results extend to a working fish heart the striking anti-adrenergic properties of CST previously reported on *in vitro* frog (Mazza et al., 2008) and rat (Angelone et al., 2008) hearts. This is of particular interest in relation to the recognition that stress in teleost fish causes an almost instant activation of the sympathetic nervous system and an immediate release of catecholamines, principally from the chromaffin cells located in the posterior cardinal vein in the region of the head kidney (Fabbri et al., 1998). Therefore, it is likely that CST, if released under stressful conditions (exercise, strenuous locomotory activity, social status effects, etc), plays a cardio-protective role by increasing the sensitivity of the Frank–Starling mechanism and hence myocardial contractility.

These data strengthen the notion that CgA is a ubiquitous prohormone precursor of inhibitory proteins that might synergistically counteract excessive excitatory stimulations of stressed organs (Mahata et al., 1997; Koeslag et al., 1999), including the heart (see Angelone et al., 2008). We believe our results can provide an insight to reconsider counter-regulatory strategies in vertebrate cardiac homeostasis.

LIST OF ABBREVIATIONS

AR	adrenoceptor
β-AR	beta-adrenoreceptor
bCgA	bovine $CgA_{344-364}$
CgA	chromogranin A
cGMP	cyclic guanosine mono-phosphate
CST	catestatin
eNOS	endothelial NOS
f_H	heart rate
$G_{i/o}$	G protein
GC	guanylate cyclase
GTP	guanosine tri-phosphate
hCgA	human $CgA_{352-372}$
ISO	isoproterenol
L-NMMA	N^G-monomethyl-L-arginine
NO	nitric oxide
NOS	nitric oxide synthase
ODQ	1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one
p-eNOS	phospho-eNOS

PKG	protein kinase G
PTx	pertussis toxin
$\dot{Q}$	cardiac output
SERCA2a	SR-CA^{2+}ATPase pumps
sGC	soluble guanylate cyclase
SR_{59230}	3-(2-ethylphenoxy)-1-{[(1S)-1,2,3,4-tetrahydronapth-1-yl]amino}-(2S)-2-propanol oxalate salt
V_S	stroke volume
VS-1	vasostatin 1
W_S	stroke work

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