Phosphodiesterase type-2 and NO-dependent S-nitrosylation mediate the cardioinhibition of the antihypertensive catestatin

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Phosphodiesterase type-2 and NO-dependent S-nitrosylation mediate the cardioinhibition of the antihypertensive catestatin. Am J Physiol Heart Circ Physiol 302: H431–H442, 2012. First published November 4, 2011; doi:10.1152/ajpheart.00491.2011.—The chromogranin A (CHGA)-derived peptide catestatin (CST; hCHGA352–372) is a noncompetitive catecholamine-release inhibitor that exerts vasodilator, antihypertensive, and cardioprotective actions. We have shown that CST directly influences the basal performance of the vertebrate heart where CST dose dependently induced a nitric oxide-cGMP-dependent cardioprotective and counteracted the effects of adrenergic stimulation through a noncompetitive antagonism. Here, we sought to determine the specific intracardiac signaling activated by CST in the rat heart. Physiological analyses performed on isolated, Langendorff-perfused cardiac preparations revealed that CST-induced negative inotropism and lusitropism involve β2/β3-adrenergic receptors (β2/β3-AR), showing a higher affinity for β2-AR. Interaction with β3-AR activated phosphatidylinositol 3-kinase/endothelial nitric oxide synthase (eNOS), increased cGMP levels, and induced activation of phosphodiesterases type 2 (PDE2), which was found to be involved in the antiadrenergic action of CST as evidenced by the decreased cAMP levels. CST-dependent negative cardiomodulation was abolished by functional denudation of the endothelium with Triton. CST also increased the eNOS expression in cardiac tissue and human umbilical vascular endothelial cells, cells confirming the involvement of the vascular endothelium. In ventricular extracts, CST increased S-nitrosylation of both phospholamban and β-arrestin, suggesting an additional mechanism for intracellular calcium modulation and β-adrenergic responsiveness. We conclude that PDE2 and S-nitrosylation play crucial roles in the CST regulation of cardiac function. Our results are of importance in relation to the putative application of CST as a cardioprotective agent against stress, including excessive sympathetic chroomaffin overactivation.

CST signal could be transduced to induce the myocardial and cardioprotective effects against ischemia/reperfusion-induced contractile dysfunction and cell death in Langendorff-perfused rat heart and in isolated ventricular cells. On the whole, these data point to CST as an endogenous cardiac inotropic, lusitropic, and coronary modulator, which, in addition to its hypotensive vasodilatory profile, is able to counteract the excessive systemic and/or intracardiac excitatory stimuli (e.g., catecholamines and ET-1).

In our previous work (2), we showed that CST acts through β2-adrenergic receptor (AR)-Gβγ-protein-NO-cGMP signaling pathways to exert its negative modulation, thereby implicating a mechanistic link between the antihypertensive property of CST and the known sympathoinhibitory influence of NO in cardiovascular functions. However, it is not known in detail how the CST signal could be transduced to induce the myocardial and half-life (2).

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coronary responses. Therefore, we studied here the peptide interactions at plasma-membrane receptor level and the successive downstream steps of the decoding process. We show the peptide interactions with the G-protein-coupled β-AR and ET-1 subtypes, as well as their functional link with a multi-branched NO-dependent pathway. The latter includes CST-induced phosphorylation of endothelial nitric oxide synthase (eNOS) and neural NOS (nNOS), as well as the consequent S-nitrosylation of phospholamban (PLN) and β-arrestin. We also demonstrate the involvement of PDE2. This rapid (PDE2 and PLN) and medium-term (β-arrestin-mediated β1-AR desensitization) decoding process appears to converge on offsetting β-sympathetic overactivity. This establishes an intriguing new mechanism of the CST action, which may be of relevance under physiological and physiopathological cardiocirculatory conditions.

MATERIALS AND METHODS

Animals

Male Wistar rats (Morini, Bologna, Italy), weighing 180–250 g, were used. Animal care, death, and experiments were done in accordance with the U.S. National Institutes of Health’s Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996) and are in accordance with the Italian law (DL 116, January 27, 1992). The scientific project was supervised and approved by the local ethical committee.

Isolated and Langendorff-Perfused Heart Preparation

Rats were anesthetized by intraperitoneal injection of ethyl carbamate (2 g/kg rat ip), and the rapidly excised hearts were immediately transferred in ice-cold buffered Krebs-Henseleit solution (KHS) for immediate cannulation through the aorta with the use of a glass cannula. Then, perfusion started at a constant flow rate (12 ml/min). To avoid fluid accumulation, the apex of the left ventricle (LV) was pierced. A water-filled latex balloon, connected to a pressure transducer (BLPR; WRI, Sarasota, FL), was inserted through the mitral valve into the LV, which allowed the recording of isovolumic contractions and continuous mechanical parameters. Another pressure transducer located just above the aorta was used to record coronary pressure (CP). The perfusion solution consisted of a modified nonrecirculating KHS containing the following (in mM): 113 NaCl, 4.7 KCl, 25 NaHCO3, 1.2 CaCl2, 1.2 KH2PO4, 11 glucose, 1.1 mannitol, and 5 Na-pyruvate (pH 7.4; 37°C; 95% O2-5% CO2). Hemodynamic parameters were assessed using a PowerLab data acquisition system and analyzed using Chart software (both purchased by ADInstruments, Oxford, UK).

Basal conditions. Cardiac performance was evaluated for inotropy by analyzing the LVP (in mmHg: an index of contractile activity) and the maximal value of the first LVP derivative (mmHg per second: an index of the maximal rate of LV contraction). Lusitropism was determined by calculating the maximal rate of LVP decline [−(LVP/dt)max; mmHg/s] and TI−t ratio between the maximal rate of LV contraction [+(LVP/dt)max] and the maximal rate of LV relaxation [−(LVP/dt)max].

Involvement of histamine receptors. To evaluate the involvement of histamine receptors (H1), cardiac preparations were perfused with 110 nM CST for 10 min and then washed out with KH. After being returned to control conditions, hearts were perfused for 10 min with Nebivolol (100 nM: a selective β1-AR antagonist) or SR59230 (100 nM: a selective β2-AR antagonist) and then with KH containing a single concentration of CST (110 nM) plus Nebivolol (100 nM) or CST plus SR59230 for an additional 10 min.

Involvement of β-ARs. To evaluate the involvement of β1-AR and β3-AR, cardiac preparations were perfused with 110 nM CST for 10 min and washed out with KH. After being returned to control conditions, hearts were perfused for 10 min with Nebivolol (100 nM: a selective β1-AR antagonist) or SR59230 (100 nM: a selective β2-AR antagonist) and then with KH containing a single concentration of CST (110 nM) plus Nebivolol (100 nM) or CST plus SR59230 for an additional 10 min.

To evaluate the relative potency of CST towards β2-AR and β3-AR, dose-response curves of Nadolol (1 to 200 nM: a selective β2-AR antagonist) or SR59230 (1 to 200 nM: a selective β3-AR antagonist; Refs. 75, 3) were generated in the presence of a single concentration of CST (110 nM). The concentration-response curves of the LVP variation induced by CST plus Nadolol or SR59230 were fitted using GraphPad Prism 4.02. This provided the −log of the concentration (in M), which induced the 50% inhibition (IC50) of CST plus Nadolol or SR59230.

Role of activated phosphatidylinositol 3-kinase activity on basal performance. The role played by activated phosphatidylinositol 3-kinase (PI3K) in the CST action mechanism was analyzed by using wortmannin or LY294,002 (LY), a selective PI3K inhibitors. Cardiac preparations were perfused with CST (110 nM) for 10 min and washed out with KHs and, after their return to control conditions, each heart was perfused with KHS containing wortmannin (100 nM) or LY (100 nM) for another 10 min. Then hearts were exposed to CST (110 nM) plus wortmannin (100 nM) or LY (100 nM).

Contribution of endothelial NOS, nNOS, and inducible NOS. To establish the contribution of endothelial NOS (eNOS), cardiac preparations, perfused with CST (110 nM) for 10 min and washed out with KHS, were perfused for 10 min with N(5)-(1-imino-3-butenyl)-L-ornithine (L-NIO; 10 μM), which is a selective eNOS inhibitor. After being washed out with KHS and returned to control conditions, each heart was perfused with KHS containing a single concentration of CST (110 nM) plus L-NIO (10 μM) for an additional 10 min. To check the association of nNOS, cardiac preparations, perfused with CST (110 nM) for 10 min and washed out with KHS, were perfused for 10 min with vinlyl-L-NIO (100 nM), a selective nNOS inhibitor. After being washed out with KHS and returned to control conditions, each heart was perfused with KHS containing a single concentration of CST (110 nM) plus vinlyl-L-NIO (100 nM) for an additional 10 min. To verify the involvement of inducible NOS (iNOS), cardiac preparations, perfused with CST (110 nM) for 10 min and then washed out with KHS, were perfused for 10 min with 1400W (10 μM), a selective iNOS inhibitor. Preparations were then washed out with KHS, and after they were returned to control conditions, each heart was perfused with KHS containing a single concentration of CST (110 nM) plus 1400W (10 μM) for an additional 10 min.

Endothelial contribution. To evaluate the coronary endothelium involvement, coronary artery endothelial dysfunction was achieved by a brief perfusion with Triton X-100 (1:200 dilution) administered after 30 min of equilibration. Triton X-100 infusion was equivalent to 1% of the flow rate delivered into the KH buffer immediately above the aorta for 1 s (36). Triton X-100 treatment induced an early increase in coronary pressure (of 37 ± 5.1 mmHg), which returned almost to control values after 20 min. LVP significantly decreased during Triton X-100 treatment and then completely recovered within 20 min. Subsequently, hearts were perfused for 25 min with normal KHS before the CST coronary actions were examined as described above. To verify the effectiveness of Triton X-100 treatment, hearts exposed to CST were washed out with KH and then perfused for 10 min with...
bradykinin (100 M). Only the hearts in which bradykinin did not change CP were considered (29).

Elisa Measurements of Intracellular cGMP/cAMP and Plasma CST

Acid extracts of frozen heart tissue (endocardial tissue and cardiomyocytes from atria and ventricles) used for cGMP and cAMP determinations (200–300 mg) were treated with 6% trichloroacetic acid at 0°C and centrifuged at 10,000 g for 10 min. The supernatant was extracted 3 times with 3 ml of diethyl ether saturated with water, and the aqueous phase was collected and stored at −80°C. cGMP and cAMP concentrations were measured using a commercial enzyme immunoassay (Biotrak enzyme immunoassay system; Amersham Biosciences, Piscataway, NJ).

CST concentration was measured by Elisa from plasma after extraction with C18 cartridge. In brief, plasma (300 μl) was mixed with equal volume of buffer A (1% trifluoroacetic acid in water) and centrifuged at 10,000 g for 20 min at 4°C, and the supernatant was removed to a new tube. Plasma samples were then passed through Sep-Pak C18 cartridge (WAT051910; Waters, Milford, MA) equilibrated in buffer A, for three to five times. cartridge was washed with buffer A, and the bound peptides were eluted with 3 ml of buffer B (60% acetonitrile and 1% trifluoroacetic acid). Samples were lyophilized to dryness and reconstituted in 300 μl of Elisa buffer. CST Elisa was performed using a commercial kit (S-1313; Peninsula Laboratories, Bachem, San Carlos, CA), according to manufacturer’s recommendation.

Role of PDEs in regulating the basal and the antiadrenergic effect of CST. The role of PDEs in the cardiac inotropism and lusitropism of CST was examined by perfusing the hearts, stabilized for 20 min with KHS, with CST (110 nM) for 10 min and then washed out with KHS. Cardiac preparations were then perfused with KHS enriched with erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA; 100 nM), a selective PDE2 inhibitor, and then with CST (110 nM) plus EHNA (100 nM) for 10 min.

To assess the contribution of PDEs in the antiadrenergic effect of CST, hearts were perfused for 5 min with ISO (5 μM). Then, they were washed out with KHS and subsequently perfused with KHS enriched with EHNA (100 nM), a selective PDE2 inhibitor, plus CST (110 nM). After being washed out, cardiac preparations were exposed for 5 min to CST (110 nM), plus EHNA (100 nM), plus ISO (5 nM).

Western Blotting

Human umbilical vein endothelial cell (HUVEC) cells and cardiac ventricles were used to evaluate differences in protein phosphorylation. HUVEC cells were purchased from American Type Culture Collection and grown in EGM-2 endothelium supplemented medium (Lonza) containing penicillin and streptomycin at 37°C and 5% CO2. In the first step, HUVEC cells were treated with CST (110 nM) and with CST (110 nM) plus i-NIO (100 nM) and then collected, washed twice with PBS, and lysed in ice-cold RIPA buffer (Sigma-Aldrich, Milan, Italy). Cardiac ventricles, obtained after perfusion with KHS alone and with a single concentration of CST (110 nM), were homogenized in ice-cold RIPA buffer (Sigma-Aldrich) containing a mixture of protease inhibitors (1 mmol/l aprotinin, 20 mmol/l phenylmethylsulfonyl fluoride, and 200 mmol/l sodium orthovanadate). Then homogenates were centrifuged at 200 g for 10 min at 4°C for debris removal. Protein concentration was determined using Bradford reagent according to the manufacturer’s recommendations (Sigma-Aldrich). Proteins were separated on 8% SDS-PAGE gels (for p-eNOS, p-iNOS, and p-nNOS detection), transferred to membrane, blocked with nonfat-dried milk, and incubated overnight at 4°C with polyclonal rabbit anti-phospho-eNOS, anti-iNOS, or -nNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1,000 in TBS-Tween containing 5% nonfat dry milk. Anti-rabbit secondary antibody peroxidase-linked (Santa Cruz Biotechnology) was diluted 1:2,000 in TBS-Tween containing 5% nonfat dry milk.

S-Nitrosylation Biotin Switch Assay

Biotin switch assay was used to evaluate protein S-nitrosylation. Ventricle homogenates were centrifuged at 4°C for 1 h at 25,000 g. Proteins contained both in the supernatant (cytosolic fraction) and in the pellet (membrane fraction, resuspended in homogenization buffer) were quantified with Bradford reagent. Biotin switch assay was performed as previously described (46). To detect bionitrilated proteins, samples from the biotin switch assay were separated on 15 and 10% SDS-PAGE gels, transferred to membrane, blocked with nonfat dried milk, and incubated with streptavidin-peroxidase diluted 1:5,000 for 1 h. In additional experiments, the membrane for S-nitrosylation detection was stripped and reprobed using a polyclonal rabbit anti-PLN antibody (Santa Cruz Biotechnology) and a polyclonal rabbit anti-phospho-β-arrestin-1 (Santa Cruz Biotechnology).

Immunodetection for both western blotting and biotin switch assay was performed using an enhanced chemiluminescence kit (ECL PLUS; Amersham). Autoradiograms were obtained by exposure to X-ray films (Hyperfilm ECL; Amersham). Immunoblots were digitalized and the densitometric analysis of the bands was carried out using NIH IMAGE 1.6 for a Macintosh computer based on 256 grey values (0 = white; 256 = black).

Drugs

Human CST was synthesized by the solid-phase method, using 9-fluorenylmethoxy-carbonyl protection chemistry (43). Peptide was purified to >95% homogeneity by preparative reverse-phase HPLC on C-18 silica column. Authenticity and purity of peptide were further verified by analytical chromatography (reverse-phase HPLC) and electrospray-ionization or matrix-assisted laser desorption mass spectrometry. PI3K inhibitors, wortmannin and LY294,002 (LY); β1-AR/β2-AR agonist, isoproteolen (ISO); strongest vasoconstrictor, endothelin-1 (ET-1); selective β2-AR inhibitor, SR59230; selective PDE2 inhibitor. EHNA; selective eNOS inhibitor, i-NIO; selective nNOS inhibitor, vinil-N(5)-(1-imino-3-butenyl)-L-ornithine (vinyl-l-NIO); selective iNOS inhibitor, 1400W; ET-1 receptor (ET1) antagonist, BQ-788, and Trition 100X (which damages the endothelium) were purchased from

Table 1. Cardiac parameters under basal conditions

<table>
<thead>
<tr>
<th>LVP, mmHg</th>
<th>HR, beats/min</th>
<th>EDV, mmHg</th>
<th>RPP, mmHg</th>
<th>CP, mmHg</th>
<th>Weight of Heart, g</th>
<th>Weight of Ventricle, g</th>
<th>Weight of Animals, g</th>
<th>Perfusion Pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>89 ± 3</td>
<td>280 ± 7</td>
<td>5–8</td>
<td>2.5 ± 0.1</td>
<td>104</td>
<td>2492 ± 129</td>
<td>-1663 ± 70</td>
<td>0.08 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
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Values are means ± SE. LVP, left ventricular pressure; HR, heart rate; EDV, end-diastolic LVP; RPP, rate-pressure product; +(LVdP/dr)max, maximal rate of left ventricular contraction; −(LVdP/dr)max, maximal rate of left ventricular pressure decline; Time to peak, time to peak tension of isometric twitch; HTR, half time relaxation; T−/t, ratio obtained by +(LVdP/dr)max/−(LVdP/dr)max; CP, coronary pressure.
mediate the histamine-dependent positive inotropism (48). Accordingly, to evaluate the involvement of H1 in the early positive inotropism induced by CST, we exposed the rat cardiac preparations to pyrilamine (100 nM), a selective H1 antagonist (30). We found that pyrilamine abolishes the early effects of CST.

Statistics

Data are expressed as the means ± SE. Since each heart represents its own control, the statistical significance of differences within the group was assessed using the ANOVA test (P < 0.05). Comparison between the groups was made by using a one-way ANOVA followed by the Bonferroni correction for post hoc t-tests. Differences were considered to be statistically significant for P < 0.05.

RESULTS

Isolated and Langendorff-Perfused Heart Preparation

Basal conditions. After 20 min of equilibration, the cardiac parameters were as indicated in Table 1. Endurance and stability of the preparations, analyzed by measuring the performance variables every 10 min, showed that the heart was stable up to 180 min.

Previously, by using the same type of rat heart preparation, we found that CST, after 5 min of administration, caused dose-dependent negative inotropic and lusitropic effects without heart rate modifications. These effects appeared starting from 11 until 165 nM (2). We also found that, under basal conditions, CST (11 to 165 nM) increases coronary pressure and counteracts the ET1-dependent coronary constriction (2).

We measured CST values in rat plasma. CST concentrations are 2.53 ± 0.11 nM. In the present study, experiments were performed using a peptide concentration (110 nM), which is known that the rat myocardium expresses H1 receptors, which

<table>
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<tr>
<th>Inhibitor</th>
<th>Biological Activity</th>
<th>LVP, Δ%</th>
</tr>
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<tbody>
<tr>
<td>Pyrilamine (100 nM)</td>
<td>Selective H1 inhibitor (30)</td>
<td>−2.17 ± 1.11%</td>
</tr>
<tr>
<td>Nebivolol (100 nM)</td>
<td>Selective β1-AR antagonist (8)</td>
<td>−4.7 ± 2.11%</td>
</tr>
<tr>
<td>Nadolol (100 nM)</td>
<td>Selective β2-AR antagonist (3)</td>
<td>−3.78 ± 1.9%</td>
</tr>
<tr>
<td>SR59230 (100 nM)</td>
<td>Selective β3-AR antagonist (53)</td>
<td>5.16 ± 2.8%</td>
</tr>
<tr>
<td>BQ-788 (100 nM)</td>
<td>ETα1 antagonist (34)</td>
<td>3.8 ± 1.8%</td>
</tr>
<tr>
<td>Wortmannin (100 nM)</td>
<td>PI3K inhibitor (61)</td>
<td>5.1 ± 3.2%</td>
</tr>
<tr>
<td>LY294,002 (100 nM)</td>
<td>Selective PI3K inhibitor (15)</td>
<td>4.31 ± 2.2%</td>
</tr>
<tr>
<td>1400W (10 μM)</td>
<td>Selective INOS inhibitor (77)</td>
<td>−3.28 ± 1.8%</td>
</tr>
<tr>
<td>L-NIO (10 μM)</td>
<td>Selective eNOS inhibitor (65)</td>
<td>−5.15 ± 2.38%</td>
</tr>
<tr>
<td>Vinyl-L-NIO (100 nM)</td>
<td>Selective nNOS inhibitor (66)</td>
<td>−4.12 ± 2.3%</td>
</tr>
<tr>
<td>EHNA (100 nM)</td>
<td>Selective PDE2 inhibitor (28)</td>
<td>3.64 ± 1.8%</td>
</tr>
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LVP, left ventricular pressure. L-NIO, N(5)-(1-imino-3-butenyl)-L-ornithine; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; AR, adrenergic receptor; ETα1, endothelin-1 receptor type B; PI3K, phosphatidylinositol 3-kinase; INOS, eNOS, and nNOS, inducible, endothelial, and neuronal nitric oxide synthase; PDE2, phosphodiesterases type 2. Numbers in parenthesis indicate reference numbers.

Sigma Chemical (St. Louis, MO). All drug-containing solutions were freshly prepared before experimentation.
positive inotropic effect, while unchanging the CST-induced negative effect (data not shown).

β2-AR and β3-AR Mediate the CST Negative Inotropic and Lusitropic Effects

Previously, we (2) demonstrated that CST-dependent inotropic and lusitropic effects are mediated by Gi/o proteins, without involving cholinergic receptors.

In the present study, we analyzed whether β2-AR and β3-AR, which are known to be responsible for negative inotropism, contribute to the CST-mediated effects. We found that chemical blockade of β3-AR by SR59230 (a specific β3-AR antagonist) either abolished the cardiac effects of CST on LVP (Fig. 1A), +LVdP/dt max and −LVdP/dt max or partially reduced T/t (Fig. 1A). Chemical inhibition of β1-AR by Nebivolol (100 nM: selective β1-AR antagonist), however, failed to modify CST-induced effects on negative LVP, +LVdP/dt max and −LVdP/dt max, indicating that β1-AR is not involved in CST cardiac signaling (data not shown). To evaluate the relative contribution of either β2-AR or β3-AR, we have determined IC50 in presence of increasing concentration of either Nadolol (β2-AR antagonist), or SR59230 (β3-AR antagonist) in response to a fixed concentration of CST (110 nM) which confirmed that the CST-induced negative inotrop...
The minimum efficacy concentration of Nadolol and SR59230, which is able to modify the CST-dependent effects, is 1 nM.

ETB Regulates the CST-Dependent Negative Inotropic Effect

In the frog heart, CST effect was mediated by ETB (49). Therefore, to assess the ETB involvement on the inotropic and lusitropic effects induced by CST, cardiac preparations were perfused with a specific antagonist of ETB (BQ788 at 100 nM). Results indicate that chemical inhibition of ETB caused complete abolition of the CST action (Fig. 1C).

PI3K Activation Mediates CST Action

PI3K/Akt is an important intracellular signal transduction molecule that regulates NO generation in cardiac endothelial cells and myocytes (64).

We evaluated the contribution of PI3K/Akt signal transduction pathway after its chemical blockade by wortmannin (100 nM). This substance abolished the effects of CST (110 nM) on negative inotropism and lusitropism (Fig. 2A). To confirm the involvement of PI3K/Akt signal, we used the highly specific inhibitor LY that, at 100 nM, inhibits PI3K activation (15). This is consistent with CST acting through a PI3K/Akt-dependent signal transduction pathway.

CST Induces Negative Inotropism and Lusitropism by NO Signaling

We (5) have previously shown that the NO-cGMP-protein kinase G (PKG) pathway, a key player of myocardial contrac-
tility, is involved in the CST-dependent inotropism and lusitropism (2). In the present study, we found that the CST-induced negative inotropism and lusitropism were abolished by L-NIO (a selective eNOS inhibitor; Fig. 2B) and reduced by vinyl-L-NIO (a selective nNOS inhibitor; Fig. 3A), while 1400W (a selective iNOS inhibitor) did not modify CST effects (Fig. 3B). This reveals that both eNOS and nNOS participate to
the CST cardiac effects. This was confirmed by the increased eNOS (Fig. 4A) and nNOS (Fig. 4B) phosphorylation, observed in whole hearts extracts after CST (110 nM) exposure, that disappears in the presence of specific eNOS and nNOS inhibition (Fig. 4, A and B). In contrast, in the perfused hearts iNOS phosphorylation was unaffected by CST (Fig. 4C). Of note, in HUVEC, CST-stimulated phosphorylation was abolished after chemical inhibition of eNOS by L-NIO (Fig. 4D).

These findings indicate that CST exerts negative inotropism and lusitropism through NO signaling.

**Endothelium Participates in CST Modulation**

It has been reported that the vascular endothelium is an important source of NO that plays a key role in mediating a specific intracardiac signaling involved in both the beat-to-beat and the long-term control of the contractile performance (9). The participation of the coronary vascular endothelium in the CST-dependent cardiac effects was examined after endothelial inactivation/denudation by Triton X-100 (36). We found that the detergent abolished the CST-dependent negative inotropism and lusitropism (Fig. 5), implicating an important role of the endothelium in the CST effect.

**cGMP Regulates the Cardiac Effects of CST**

Soluble guanylyl cyclase, which generates cGMP, was the first identified target for NO (37, 52). NO binding to the heme group of soluble guanylyl cyclase leads to increased conversion of GTP to cGMP, which in turn activates protein kinase G (PKG) to result ultimately in a reduction of cardiac contractility (47). Consistent with CST signaling through cGMP, we found increased cGMP content in CST-treated heart (Fig. 6A).

**PDE2 Regulates Basal and Antiadrenergic Effects of CST**

In cardiac myocytes, cGMP decreases Ca\(^{2+}\) influx through \(\mathcal{L}\)-type channels by stimulating PDE2 (cAMP phosphodiesterase; Ref. 16). To explore whether PDE2 activity is involved in the CST-induced negative inotropism, the cardiac preparations were pretreated with EHNA (100 nM; a selective inhibitor of PDE2), which abolished the negative inotropism and lusitropism induced by CST (110 nM; Fig. 6B).

We (2) have previously shown that CST acts as a non-competitive antagonist to ISO-induced adrenergic stimulation. To determine the possible PDE2 contribution in the antiadrenergic action of CST, hearts were perfused with ISO (10 nM) after treatment with the PDE2 inhibitor EHNA (100 nM) in the...
presence of CST (110 nM). PDE2 inhibition abolished the CST-induced modulation of the ISO-dependent inotropism (Fig. 7A). We also observed that CST suppressed the ISO-induced elevation of cAMP. cAMP reduction disappears also in the presence of PDE2 inhibition (Fig. 7B).

Protein S-Nitrosylation Is Involved in the Vardiac Effects of CST

In addition to the classic NO-cGMP-dependent pathway, NO regulates cardiac function also through protein S-nitrosylation, i.e., the covalent modification of a protein cysteine thiol by an NO group to generate S-nitrosothiol (71). Therefore, to determine whether the CST-induced cardiac effects require protein S-nitrosylation, we analyzed S-nitrosylation of cardiac proteins containing cysteines in homogenates of both control and CST-treated hearts. By using the biotin switch method, associated to Western Blot determination, we found that CST increased the S-nitrosylation of a proteins which correspond to the apparent molecular mass of β-arrestin (50 kDa) and PLN (monomer: 6 kDa, dimer: 12 kDa, pentamer: 30 kDa; Fig. 8, A and B).

DISCUSSION

Functional and molecular investigations have recently revealed that CST, in addition to its antihypertensive and vasoactive actions (41, 55), induces cardiosteption via direct negative inotropic and lusitropic effects on the basal heart performance and counteracts β-adrenergic stimulation and ET-1-induced positive inotropism and coronary constriction (2). Preliminary data on these cardiac actions of CST revealed the contribution of β2-AR-Gi/o protein-NO-cGMP signaling (2). The aim of the present study was to identify the downstream intracellular cascade inputs to the effectors responsible for the CST cardioactivity.

The plasma levels of CST measured in the rat are 2.53 ± 0.11 nM and are comparable to the 1.47 ± 0.06 nM detected in human plasma (57). Our results show that the CST-dependent negative inotropism and lusitropism, as well as its antiadrenergic and anti-ET-1 effects are achieved through PDE2 and NO-dependent PLN and β-arrestin S-nitrosylation, adding a relevant piece of information on the CHGA-induced natural protection of the heart against excessive sympathochromaffin over-activation, e.g., hypertensive cardiomyopathy (74). We will first discuss CST interactions at plasma membrane level and then its subsequent downstream transduction pathway.

β-Adrenergic and ET-1 Involvement

β2-AR functionally coexists with the β1-AR in the myocytes of many mammals, including rodents (82). β1-AR, coupled to Gs proteins, is responsible for positive inotropism and lusitropism, while β2-AR, mainly coupled to Gi/o proteins, is responsible for the opposite effects on contractility and relaxation (82). The heart of mammalian (23, 24, 69, 2) and nonmammalian (33, 49) vertebrates also expresses Gi/o-protein-coupled β3-AR, which induces negative inotropism and lusitropism (2, 23). Activated by physio-pathological nor-

Fig. 8. S-nitrosylation of cardiac proteins. A and B: Western blot analysis of S-nitrosylated proteins in homogenized cardiac ventricles. A: S-nitrosylation of membrane protein fraction (I) and stripped membrane (II) incubated with an anti-β-arrestin antibody showing S-nitrosylation at the migration position corresponding to β-arrestin. B: S-nitrosylation of membrane protein fraction (I) and stripped membrane (II) incubated with an anti-phospholamban (PLN) antibody showing S-nitrosylation at the migration position corresponding to the PLN as a monomer (6 kDa), dimer (12 kDa), and pentamer (30 kDa). Percent changes were evaluated as means ± SE of 3 experiments. *P < 0.05 vs. control. §P < 0.05 between groups.
adrenaline concentrations, β3-ARs counteract the effects induced by catecholamine hyperstimulation (22).

We found that CST myocardial effects involve both β2-AR and β3-AR, but not β1-AR, with a higher affinity for the first one, as evidenced by low IC50 values. We (2) previously reported that CST-dependent negative inotropism and lusitropism involve Gs/o proteins. This suggested that the CST direct negative inotropism and lusitropism could result from Gi/o proteins activation, which limits the Gi-mediated contractile effects.

On the rat heart, CST counteracts the ET-1-induced positive inotropism and lusitropism and coronary constriction (2). As suggested by data obtained in the frog heart, the ET-1-induced negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49). Since in mammals stimulation of cardiac ETB elicits an endothelium-negative inotropism is ETB-EE-NO dependent (49).
signaling that rapidly switches off adrenergic activity through PDE2, and a medium-term signaling that elicits more prolonged adrenergic counteraction by stimulating β-arrestin-mediated β1-AR desensitization.

PLN is emerging as another component of the CST cardiac scenario. It controls sarcoplasmic reticulum Ca-ATPase (SERCA2a) by a phosphorylation/dephosphorylation mechanism. When dephosphorylated, PLN inhibits SERCA2a-dependent SR Ca2+ sequestration (62). Its phosphorylation at Ser16 by PKA relieves its inhibition on SERCA2a (67). Froehlich et al. (17) showed that the PLN-SERCA2a interaction is also modulated by the Angeli’s salts-derived HNO that covalently modifies the critical thiol residues of both proteins. The consequent conformational changes relieve the inhibition of the pump. Recently, in nonmammalian hearts, S-nitrosylation of PLN has been proposed as an additional mechanism which regulates stretch-induced contractile effects (21 and references therein). In the present study, we suggest that the CST-induced NO-dependent PLN S-nitrosylation may contribute to the cardioprotection elicited by the peptide through the regulation of SR Ca2+ fluxes and thus the availability of Ca2+ for the contractile apparatus.

Conclusions

The heart is a target organ of adrenergic hyperactivation, as epitomized by increased sympathetic, endothelin-1, and reactive oxygen species stimuli that characterize the neuroendocrine scenario of the congestive cardiac failure (84). The detection of Chga (70) and its derived CST in murine cardiomyocytes (4), together with the demonstration of CST-dependant myocardio-suppressive antiadrenergic and anti-ET-1 activities, suggested a direct autocrine/paracrine cardioprotective function of the peptide (2, 6). This hypothesis is now further supported by the present study that in the rat heart proposes several rapid (PDE2 and PLN) and medium-term (β-arrestin-mediated β1-AR desensitization) regulatory switches by which CST is able to counterbalance the adrenergic-mediated increases in inotropy, thereby acting as an endogenous β-blocker (Fig. 9). This branched pathway may provide CST cardioinhibitory modulation with a wider temporal window than previously conceived, stimulating hints of physio-pathological interest, particularly in relation to perturbed cardiac homeostasis caused by prolonged enhanced sympathetic overlap.

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Disclosures

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Author Contributions


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