Human recombinant chromogranin A-derived vasostatin-1 mimics preconditioning via an adenosine/nitric oxide signaling mechanism

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2Pharmacology-Biology and 3Cell Biology, University of Calabria, Arcavacata di Rende (CS); 4Department of Physiological Sciences, University of Turin, ASO San Luigi, Orbassano; and 5Department of Biological and Technological Research, San Raffaele H Scientific Institute, Milan, Italy

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Cappello S, Angelone T, Tota B, Pagliaro P, Penna C, Rastaldo R, Corti A, Losano G, Cerra MC. Human recombinant chromogranin A-derived vasostatin-1 mimics preconditioning via an adenosine/nitric oxide signaling mechanism. Am J Physiol Heart Circ Physiol 293: H719–H727, 2007. First published April 6, 2007; doi:10.1152/ajpheart.01352.2006.—The acidic protein chromogranin A (CgA) is the precursor of several regulatory peptides generated by specific proteolytic processes. Human recombinant CgA NH2-terminal fragment STA-CgA1-78 (hrSTA-CgA1-78), containing vasostatin-1 (CgA1-76) domain, exerts a negative inotropic effect and counteracts the ß-adrenergic positive inotropic effect on the rat heart. We hypothesized an involvement of nitric oxide (NO)-dependent pathway in both cardio-depression and cardioprotection by hrSTA-CgA1-78. We also hypothesized an involvement of adenosine A1 receptor and protein kinase C (PKC) in cardioprotection by hrSTA-CgA1-78. Therefore, we evaluated whether 1) the cardioinhibition mediated by hrSTA-CgA1-78 involves the CgA1-78 proteins/NO-dependent signal transduction cascade, 2) hrSTA-CgA1-78 induces ischemic preconditioning-like protective effects on the myocardium, and 3) inhibition of NO synthase (NOS), adenosine A1 receptor, or PKC affects hrSTA-CgA1-78 protection. Using the isolated rat heart, we found that the reduction of left ventricular pressure (LVP), rate-pressure product, and maximal values of the first derivative of LVP elicited by hrSTA-CgA1-78 at 33 nM is abolished by blocking Gi/o proteins with pertussis toxin, scavenging NO with hemoglobin, and blocking NOS activity with Nω-monomethyl-L-arginine or Nω-(iminoethyl)-l-ornithine, soluble guanylate cyclase with 1H-[1,2,4]oxadiazolo-[4,3-b]quinoxalin-1-one, and protein kinase (PKG) with KT5823. Data suggest the involvement of the G1/o proteins/N0-cGMP-PKG pathway in the hrSTA-CgA1-78-dependent cardioinhibition. When given before 30 min of ischemia, hrSTA-CgA1-78 significantly reduced the size of the infarct from 64 ± 4 to 32 ± 3% of the left ventricular mass. This protective effect was abolished by either NOS inhibition or PKC blockage and was attenuated, but not suppressed, by the blockade of A1 receptors. These results suggest that hrSTA-CgA1-78 activity triggers two different pathways: one of these pathways is mediated by A1 receptors, and the other is mediated by NO release. As with repeated brief preconditioning ischemia, hrSTA-CgA1-78 may be considered a stimulus strong enough to trigger both pathways, which may converge on PKC.

vasostatin; contractility

CHROMOGRANIN A (CgA) is the major soluble product within the secretory granules of chromaffin cells of the adrenal medulla.

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hrSTA-CgA1-78-dependent β-adrenergic counteraction occurs with a functional noncompetitive type of antagonism (8).

Structure-function analyses also revealed the functional importance and the high phylogenetic conservation of domains such as CgA1-40 and CgA7-57 in both eliciting the negative inotropic effect and counteracting the β-adrenergic stimulation (11, 25, 50). Importantly, in the eel heart, hrSTA-CgA1-78 activated the nitric oxide-cGMP-protein kinase G (NO-cGMP-PKG) pathway (25). Whether hrSTA-CgA1-78 can also activate this pathway in mammals remains to be elucidated.

Hearts exposed to brief, sublethal ischemic insults are more resistant against subsequent, prolonged ischemia. This phenomenon of ischemic preconditioning has been described in all the tested species (see e.g., Refs. 9, 36, 52). The brief cycles of preconditioning ischemia cause the release of several autacoids, which trigger multiple pathways leading to protection. Most of them couple indirectly to protein kinase C (PKC) via coids, which trigger multiple pathways leading to protection. Preconditioning ischemia cause the release of several autacoids, which trigger multiple pathways leading to protection. This pathway in mammals remains to be elucidated.

The present study was designed to investigate in the isolated rat heart whether 1) the cardioinhibition mediated by hrSTA-CgA1-78 requires activation of the G_{60} proteins/NO-dependent signal transduction system; 2) hrSTA-CgA1-78 induces ischemic preconditioning-like myocardial protective effects; or 3) inhibition of adenosine A_{1} receptor, NOS, or PKC affects hrSTA-CgA1-78 protection.

MATERIALS AND METHODS

Animals

Male Wistar rats (Morini, Bologna, Italy) weighing 300–400 g were housed three per cage in a ventilated cage rack system under standard conditions. Animals had food and water access ad libitum. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health [DHEW Publication No. (NIH) 85-23, Revised 1996, Office of Science and Health Reports, DRR/NHI, Bethesda, MD 20205] and is in accordance with the Italian ethical guidelines (DL 111, 27 January 1992). The local ethical committee approved the project.

Isolated Heart Preparation

Rats were anesthetized with ethyl carbamate (2 g/kg rat, intraperitoneally). The hearts were rapidly excised and transferred in ice-cold buffered Krebs-Henseleit solution (KH). The aorta was immediately cannulated with a glass cannula and connected with the Langendorff apparatus to start perfusion at a constant flow rate of 12 ml/min as previously described (8). Briefly, to avoid fluid accumulation, the apex of the left ventricle (LV) was pierced. A water-filled latex balloon, connected to a BLPR gauge (WRI), was inserted through the mitral valve into the LV to allow isovolumic contractions and to continuously record mechanical parameters. Coronary pressure was recorded using another pressure transducer connected with the perfusion line just above the aorta. The perfusion solution consisted of a modified non recirculating KH solution containing (in mM) 113 NaCl, 4.7 KCl, 25 NaHCO_{3}, 1.2 MgSO_{4}, 1.8 CaCl_{2}, 1.2 KH_{2}PO_{4}, 11 glucose, 1.1 mannitol, and 5 Na-pyruvate (pH 7.4, 37°C, 95% O_{2}-5% CO_{2}). Hemodynamic parameters were assessed using a PowerLab data acquisition system and analyzed using Chart software (both purchased from ADInstruments, Basile, Italy).

Experimental Protocols

The Langendorff-perfused rat heart performance was evaluated by analyzing the left ventricular pressure (LVP; in mmHg), which is an index of the contractile activity, the rate-pressure product (RPP; heart rate × LVP, in 10^{4} mmHg·beats·min^{-1}), used as an index of cardiac work (15), and maximal values of the first derivative of LVP ([dLVP/dt]_{max}; in mmHg/s), which indicates the maximal rate of LV contraction. The mean coronary pressure (CP; in mmHg) was calculated as previously described (8). RPP was not calculated for the hearts of groups 7–15, which were kept at a constant rate (280 beats/min) throughout the entire time course of the experiments. A schematic representation of the experimental protocols is shown in Fig. 1.

Effects of hrSTA-CgA1-78 on Contractile Function

All cardiac preparations, stabilized for 20 min with KH solution, were perfused with 33 nM hrSTA-CgA1-78 for 10 min and then washed out with KH solution. After returning to control conditions, the hearts were randomly assigned to one of the following groups (groups 1–6, Fig. 1A), which received for another 10 min KH solution containing the indicated antagonist: group 1 (n = 5), the NO scavenger hemoglobin (Hb; 10 µM); group 2 (n = 6), the nonspecific NOS inhibitor N^6-monomethyl-L-arginine (L-NMMA; 100 µM); group 3 (n = 6), the selective endothelial NOS (eNOS) inhibitor N^2-(iminoethyl)-L-ornithine (L-NIO; 10 µM); group 4 (n = 5), the soluble guanylate cyclase (GC) inhibitor 1H-[1,2,4]oxadiazole-[4,4,4]-aquinooxaline-1-one (ODQ; 10 µM); group 5 (n = 5), the protein kinase G (PKG) blocker KT5823 (100 nM); and group 6 (n = 4), the G_{60} protein inhibitor pertussis toxin (PTX; 10 µM). The hearts of each group were then exposed to the specific drug plus 33 nM hrSTA-CgA1-78.

The concentration of hrSTA-CgA1-78 was chosen on the basis of a previous study (8). Preliminary experiments (data not shown) performed by the repetitive exposure of each heart to hrSTA-CgA1-78 revealed the absence of desensitization.

Effects of hrSTA-CgA1-78 on Ischemia-Reperfusion Injury

The hearts of the remaining groups (groups 7–15; Fig. 1B) were paced at 280 beats/min to prevent changes in heart rate from altering the extension of infarct size. After 20 min of stabilization with KH solution, cardiac preparations underwent 30 min of global ischemia, followed by 120 min of reperfusion (I/R protocol). In group 7 (n = 9), the hearts were exposed to I/R only. In group 8 (n = 7), before I/R, the hearts also underwent ischemic preconditioning consisting of three cycles of 3 min of global ischemia, separated from each other by 5 min of reperfusion. In group 9 (n = 7), after stabilization, hrSTA-CgA1-78 (80 nM) was infused for 19 min before I/R. In group 10 (n = 8), hrSTA-CgA1-78 was given for 19 min as in group 9, except to bracket the hrSTA-CgA1-78 infusion, the NOS inhibitor l-NIO (10 µM) was also infused 5 min before and 5 min after the infusion of hrSTA-CgA1-78 for a total of 29 min (hrSTA-CgA1-78 + l-NIO). In group 11 (n = 7), the NOS inhibitor was replaced by the A_{1} adenosine receptor blocker 1,3-dipropargyl-8-cyclopenthynanthine (DCP; 5 µM), and in group 12 (n = 5), the PKC inhibitor chelerythrine (CHE; 5 µM) was infused in lieu of DCPX. As for groups 13–15, these were additional control groups in which experiments were performed using either l-NIO (group 13; n = 4), DCPX (group 14; n = 4), or CHE alone (group 15; n = 4) to test a possible effect of these compounds on the infarct size. Since in groups 10–12 these antagonists were
infused for 29 min, each inhibitor in groups 13–15 also was infused for 29 min before ischemia in the absence of hrSTA-CgA_{1-78}. After the third cycle of preconditioning ischemia, as well as after hrSTA-CgA_{1-78} and/or antagonist treatment, the hearts were washed with KH solution for 10 min before the I/R protocol was started.

The concentration of hrSTA-CgA_{1-78} was chosen on the basis of a preliminary dose-response curve (from 20 to 80 nM) as the dose that induced the highest infarct size reduction. All antagonist concentrations were selected on the bases of the results of preliminary dose-response curves as the first effective dose that did not significantly affect the cardiac performance (data not shown).

### Analysis of Lactate Dehydrogenase Production

Since in isolated rat hearts, ischemic preconditioning is known to reduce the production of lactate dehydrogenase (LDH) during reperfusion (Ref. 37 and references therein), the release of this enzyme was tested. Samples of coronary effluent (2 ml) were withdrawn with a catheter inserted into the right ventricle via the pulmonary artery. Samples were taken immediately before ischemia and at 3, 6, 10 and 20 min of reperfusion. Thereafter, samples were collected every 20 min until the end of reperfusion. LDH release was measured as previously described (37, 41). Data are expressed as cumulative values for the entire reperfusion period.

### Infarct Size Assessment

After the end of reperfusion, the hearts in groups 7–15 were rapidly removed from the perfusion apparatus. The LV was then isolated from the other parts of the heart and dissected into circumferential slices. After 20 min of incubation at 37°C in a 0.1% solution of nitro blue tetrazolium in phosphate buffer, white unstained necrotic tissue was accurately separated from red-stained viable tissue by a blind independent observer (37, 40, 41). The weights of the necrotic and viable tissues were determined, and the necrotic mass was expressed as a percentage of the total mass of the LV. Although in our experimental model the whole heart underwent I/R, only the LV had a fixed volume and preload throughout the experiment. This is the reason why only the LV was studied as risk area.

### Statistics and Analysis of Data

Data are means ± SE.
**Myocardial Depression by hrSTA-CgA1-78**

The performance variables were measured every 10 min, up to 180 min. In the control group, perfusion with KH buffer alone at isovolumic conditions provided the following basal parameters, measured after 20 min of equilibration: \( LVP, 90 \pm 2.9 \text{ mmHg}; \) RPP, \( 2.57 \pm 0.12 \times 10^2 \text{ mmHg} \cdot \text{beats} \cdot \text{min}^{-1}; \) \(+\text{LVdP/dt})_{\text{max}}, 2.492 \pm 0.129 \text{ mmHg/s}; \) and CP, \( 69 \pm 4.2 \text{ mmHg}. \) hrSTA-CgA1-78 at 33 nM induced a negative inotropic effect on the isolated and perfused rat heart, as revealed by the significant reduction of \( LVP, RPP, \) and \(+\text{LVdP/dt})_{\text{max}} \) (Fig. 2). However, CP was not affected.

hrSTA-CgA1-78-induced reduction of \( LVP, RPP, \) and \(+\text{LVdP/dt})_{\text{max}} \) was abolished by both the removal of NO with Hb and by the NOS inhibition with \( \text{L-NMMA} \) and \( \text{L-NIO} \) (Fig. 2). It also was abolished by pretreatment with ODQ, as well as by the inhibition of PKG with KT5823 (Fig. 2). These results indicate an involvement of the NO-cGMP-PKG signal transduction pathway in the hrSTA-CgA1-78-mediated responses.

To verify the involvement of G\(_{\alpha}\) and G\(_{\beta/\gamma}\) proteins in the negative inotropic effect mediated by hrSTA-CgA1-78, we pretreated the cardiac preparations with PTx, which uncouples signal transduction between several families of receptors and G\(_{\alpha}\) or G\(_{\beta/\gamma}\) proteins (Ref. 4 and references therein). Although pretreatment with PTx alone did not significantly change basal cardiac performance (data not shown), it abolished the negative inotropic effect induced by hrSTA-CgA1-78 at 33 nM (Fig. 3).

**Myocardial Protection by hrSTA-CgA1-78**

In the absence of any treatment (group 7), after 30 min of global ischemia and 2 h of reperfusion, the total infarct size was \( 64 \pm 4\% \) of the LV mass. Ischemic preconditioning (group 8) significantly \((P < 0.005 \text{ vs. group 7})\) reduced the infarct size to \( 35 \pm 5\% \) of the LV (Fig. 4A).

Similar to ischemic preconditioning, the infusion of hrSTA-CgA1-78 at a concentration of 80 nM for 19 min before I/R (group 9) reduced the infarct size to \( 32 \pm 3\% \) \((P < 0.005 \text{ vs. group 7})\). The NOS inhibitor \( \text{L-NIO} \) (group 10) completely abolished hrSTA-CgA1-78 protection so that the infarct size was not significantly different from that of group 7 (66 \pm 5 vs. 64 \pm 4\%).

The A\(_1\) adenosine receptor blocker DCPX partially suppressed the protective effect of hrSTA-CgA1-78. In fact, the infarct size was significantly higher than that of group 9 (48 \pm 5 vs. 32 \pm 3\%; \( P < 0.01 \)) but was significantly lower than that of group 7 (64 \pm 4\%; \( P < 0.05 \)).

The PKC blocker CHE completely abolished hrSTA-CgA1-78 protection (group 12) so that the infarct size was not significantly different from that of group 7 (76 \pm 4 vs. 64 \pm 4\%).
When the changes in infarct size in the various groups are expressed as percentages of the control group 7 (normalization), the normalized data (Fig. 4B) show the same significance observed for the absolute data. In the absence of any pretreatment, the release of LDH during reperfusion was 1,343 ± 161 U/g wet wt (group 7) (Fig. 5A). When absolute data are compared, LDH release was significantly reduced only after pretreatment with hrSTA-CgA1-78 alone (group 9) and with hrSTA-CgA1-78 plus A1 receptor blocker (group 11) (P < 0.05 vs. group 7 for both) (Fig. 5A). It is likely that in the other groups, the dispersion of the data prevented any statistical difference, which is not unusual when LDH release is measured in different experiments (47). However, when the changes of LDH release in the various groups are expressed as percentages of the release after I/R only (normalization; Fig. 5B), a significant decrease is observed in ischemic preconditioning (group 8) and in all cases in which hrSTA-CgA1-78 was infused (groups 9–12).

In the absence of hrSTA-CgA1.78, the infarct size and LDH release were not reduced with respect to the control (group 7) (Figs. 4 and 5).

**DISCUSSION**

**Methodological Considerations**

We utilized the human recombinant VS-1-containing peptide on the rat heart. It has been reported that the proteolytic processings of CgA are likely to be different in the human and rat (16, 48). However, the elevated structural homology between human and rat sequences (i.e., 82% homology of CgA17-38), together with the negative inotropy exerted by hrSTA-CgA1.78 on the rat heart (Refs. 8, 14, and present study), suggests that the inotropic activity of the human and rat peptides is highly conserved. It also has been suggested that the rat heart produces the fragment CgA1-64 (32). Preliminary functional studies carried out by our research group using rat CgA1-64 have shown that this fragment exerts inotropic effects that are similar to those obtained with hrSTA-CgA1-78 (unpublished observations).

![Fig. 3. Effects of hrSTA-CgA1-78 (33 nM) alone and hrSTA-CgA1-78 in the presence of PTx (0.01 nM) on LVP, RPP, and +LVdp/dtmax on the rat isolated and Langendorff-perfused heart. Percent changes were evaluated as means ± SE of 4 experiments for each peptide concentration. Significance of difference from control values (t-test): *P < 0.05; **P < 0.005 vs. group 7. Comparison between groups (ANOVA, Duncan’s test): §P < 0.05.](http://ajpheart.physiology.org/)

![Fig. 4. Protective effect of hrSTA-CgA1.78 against the extension of an infarct size. A: absolute data of infarct size. B: normalized data of infarct size. Values are means ± SE. Significance of difference from control values (unpaired t-test): *P < 0.05, **P < 0.005 vs. group 7; #P < 0.01; ##P < 0.005 vs. group 9. LV, left ventricle.](http://ajpheart.physiology.org/)
Therefore, the lack of species specificity of hrSTA-CgA1-78 on the isolated and Langendorff-perfused rat heart suggests that this model may be utilized for studying the effect of CgA NH2-terminal fragments on mammalian heart. Moreover, by reducing the number of independent variables to a reasonable minimum, this model can be used to study the acute effects of substances on heart contractility (e.g., Refs. 8, 13, 43, 50) and the effects of I/R on infarct size (e.g., Refs. 17, 37, 40, 41). Infarct size is usually detected with the nitro blue tetrazolium technique. LDH release is often correlated with infarct size. The correlation, however, is not always significant. We believe that the discrepancy does not weaken the data obtained with LDH. In the mammalian heart, the NOS-NO-cGMP-PKG system plays a key role in mediating specific intracardiac signaling involved in the control of the contractile performance. All three NOS isoforms (namely, eNOS, iNOS, and neuronal NOS) are involved in the control of the contractile performance. All three

Myocardial Depression

Using the same rat heart preparation, our group recently demonstrated that hrSTA-CgA1-78 transiently depresses myocardial function in a dose-dependent manner (8). In fact, during intracoronary infusion, hrSTA-CgA1-78 reduces LVP and RPP. It also counteracts the β-adrenergic stimulation induced by isoproterenol with a functional noncompetitive antagonism, suggesting a role as stabilizer of adrenergic tone in cardiovascular homeostasis (8). It should be noticed that although the in vivo serum levels of the precursor CgA are 1–4 nM (20), the first hrSTA-CgA1-78 concentration that affects the inotropic behavior of our model is 33 nM (8). Accordingly, in the present investigation, this concentration was used to analyze the mode of action of the peptide.

We observed that the reduction of LVP, RPP, and + (LVdP/dt)max induced by hrSTA-CgA1-78 at 33 nM was abolished by either scavenging NO with Hb or blocking NO activity with L-NMMA (unspecific) and L-NIO (e-NOS selective) or by inhibiting soluble GC with ODQ and cGMP-dependent protein kinase with KT5823, an inhibitor structurally unrelated to cGMP. These data are all consistent with an NO-dependent mechanism underlying the hrSTA-CgA1-78-induced negative inotropic action in the rat heart.

In the mammalian heart, the NOS-NO-cGMP-PKG system plays a key role in mediating specific intracardiac signaling involved in the control of the contractile performance. All three NOS isoforms (namely, eNOS, iNOS, and neuronal NOS) are expressed in different myocardial cell types, showing a well-defined subcellular compartmentalization with colocalized effectors. In rat ventricular myocytes, the NOS-produced NO, by targeting soluble GC, and thus PKG, negatively affects contractility by reducing L-type Ca2+ current (2, 30) and by phosphorylating troponin I, thus reducing the affinity of troponin C for calcium and depressing contractility (21). We propose that both L-type Ca2+ current reduction and PKG-medi-
ated myofilament desensitization to Ca\(^{2+}\) may account for hrSTA-CgA\(_{1-78}\)-induced negative inotropy. In addition, activation of PKG by NO-dependent cGMP induces the phosphorylation of \(\alpha_1\)-subunit of the PTX-sensitive \(G_{i/o}\) proteins, and this strengthens the inhibition operated on L-type Ca\(^{2+}\) current (2). This PKG-induced action on \(G_{i/o}\) proteins, on one hand, negatively affects adenyl cyclase, causing a decrease of cAMP levels; on the other hand, it stimulates eNOS-dependent NO production (32, 42). The functional consequence of this concerted cascade is inhibition of contractility. Interestingly, in our experiments we observed that the hrSTA-CgA\(_{1-78}\)-induced negative inotropic effect is blocked not only by inhibition of the NOS-NO-cGMP-PKG system but also by the selective impairment of \(G_{i/o}\) proteins by PTX. Further study is needed to highlight the mechanism by which the peptide interacts with these multiple intracellular effectors. Whatever the route utilized by hrSTA-CgA\(_{1-78}\) to affect intracellular signaling, i.e., via either a still unknown receptor or lipophilic interaction with the membrane (Ref. 18 and references therein), it is reasonable to hypothesize that it exerts its negative inotropic influence by modulating NOS activity, through either a direct control of e-NOS or the modulation of \(G_{i/o}\) proteins. Alternatively, or additionally, hrSTA-CgA\(_{1-78}\) may act at the level of downstream effectors, such as PKG, thus controlling intracellular Ca\(^{2+}\) homeostasis and utilization. PKG may also feedback on \(G_{i/o}\) proteins, creating a circuit of interactions that globally converge to depress contractility.

**Myocardial Protection**

The involvement of NO-cGMP-PKG pathway in the negative inotropic effect suggests the possibility that hrSTA-CgA\(_{1-78}\) exerts a protective effect against the extension of myocardial infarctions. Therefore, we tested whether hrSTA-CgA\(_{1-78}\) might induce a preconditioning-like effect if given before I/R. Indeed, the administration of a low dose of hrSTA-CgA\(_{1-78}\) before I/R reduced the infarct size. This protective effect was suppressed by eNOS inhibition and ~50% reduced by the blockade of A1 receptors, indicating that at least two different pathways may be hypothesized as possible mediators of cardioprotection (Fig. 6).

The role of NO in preconditioning has been demonstrated in several investigations. NO, in fact, is responsible for the activation of soluble GC and the production of cGMP, which, via the intervention of PKG, induces the opening of the mitochondrial ATP-sensitive K\(^{+}\) channels, which is then followed by activation of PKC and other kinases via reactive oxygen species production (52).

A second pathway may imply the participation of the adenosine A1 receptor in the protection. In fact, there is evidence that adenosine A1 receptor activation with the activation/translocation of PKC (Ref. 52 and references therein) may trigger preconditioning and that in the rat heart, adenosine and opioid receptors are tightly coupled (38, 39). Based on these observations and the effect of A1 receptor blockade, we hypothesize an involvement of adenosine pathway in the protective effects seen with hrSTA-CgA\(_{1-78}\). Although the mechanism of this involvement is not known, we can argue that, similarly to opioids, hrSTA-CgA\(_{1-78}\) somehow interacts with adenosine receptors.

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**Fig. 6.** Diagram of mechanisms of hrSTA-CgA\(_{1-78}\)-induced cardioprotection showing the 2 proposed pathways (adenosine dependent and adenosine independent). It is likely that hrSTA-CgA\(_{1-78}\) can activate endothelial NOS (eNOS) directly or via adenosine receptors (AdoR). The preponderance of NO pathway also may be due to the fact that NO may derive from both endothelium and cardiomyocyte eNOS. The line interruptions indicate intermediate steps that are reported in the DISCUSSION. For further explanation, see text.

It also has been shown that activation of A2a adenosine receptors can cause the release of NO from endothelial cells and cardiomyocytes (16, 23, 24, 34, 45, 46), thus reinforcing the NO-triggered pathway. As a matter of fact, it was recently shown that opioids also interact with A2a receptors (51) and that the hrSTA-CgA\(_{1-78}\) cardiodepressive effect is mainly mediated by endothelial release of NO (14).

Although many studies seem to indicate that each of the two pathways (adenosine-dependent and adenosine-independent pathway) are sufficient per se to induce myocardial protection, several investigations have reported that the blockade of one pathway only may result in the removal of the protection (36, 52). This was not the case in our study, where the blockade of A1 receptors, unlike the inhibition of NOS, reduced, but did not suppress, the protective effect of hrSTA-CgA\(_{1-78}\). It is then likely that the release of NO in a large quantity, directly and/or via adenosine receptors, is the predominant effect of the peptide. This is consistent with the other observation of our study, which indicates that the administration of hrSTA-CgA\(_{1-78}\) reduces myocardial contractility, an effect that is achieved only by high concentrations of NO, whereas low concentrations induce the opposite effect (28).

Our findings also are consistent with the results of other studies reporting that A1 adenosine receptor antagonists can abolish the cardioprotective effect of two cycles of ischemic preconditioning, whereas they do not abolish the protective effects of strong stimuli such as four cycles of ischemic preconditioning (5, 29). It is likely that hrSTA-CgA\(_{1-78}\) may represent a strong protective stimulus that cannot be completely blocked by A1 receptor antagonist. The fact that CHE, a PKC antagonist, fully prevented the infarct-sparing effect of hrSTA-CgA\(_{1-78}\) suggests that PKC activation is a point of convergence of adenosine-independent and adenosine-dependent pathways activated by hrSTA-CgA\(_{1-78}\).

In summary, hrSTA-CgA\(_{1-78}\), like opioids, activates both an adenosine A1 receptor-dependent and a NOS-dependent pathway. Whereas the blockade of the former pathway blunted, but did not suppress, the protective effect of hrSTA-CgA\(_{1-78}\), the
inhibition of either NOS or PKC totally prevented the infarct-sparing effect of hrSTA-CgA1-78. These results suggest that hrSTA-CgA1-78 may represent a strong protective stimulus that activate a number of pathways that converge on NOS and PKC.

However, when the changes in LDH release in the various groups were expressed as percentages of the release after I/R only (normalization; Fig. 5B), a significant decrease was observed in ischemic preconditioning (group 8) and in all cases in which hrSTA-CgA1-78 was infused (groups 9–12). The more robust index of cardiac protection is infarct size reduction. In addition, the release of LDH during reperfusion could be taken as an additional index of the injury extent. Thus we expected that in each ischemic group (groups 7–15), LDH release would have changed in parallel with the changes in infarct size. This was not the case. It is likely that the large dispersion of data, which is not unusual for LDH release, prevented any statistical significance. Thus we tried to compare the various data as percent changes of the data of group 7, related to I/R only. Surprisingly, after such a normalization, a significant percent reduction of LDH release was observed in the various groups treated with hrSTA-CgA with respect to the control (group 7), even when the infarct-sparing effect of the peptide was prevented by the inhibition of NO release and by the blockade of PKC activation/translocation. It is also interesting that the blockade of A1 receptors, whose effect was lower than that of NO inhibition in impairing hrSTA-CgA1-78 protection, was characterized by a release of LDH that was significantly lower than in group 7 even in the absence of normalization. This finding seems to indicate that when the activity of hrSTA-CgA1-78 is only partially prevented, LDH release is still reduced, further supporting the idea that hrSTA-CgA1-78 may represent a strong protective stimulus. Yet, pretreatment with antagonists alone (groups 13–15) did not affect both infarct size and LDH release. It is noteworthy that statistic analysis of infarct size gives the same results with absolute and normalized data.

In the rat heart, hrSTA-CgA1-78 activates a cascade of signals, which involves the NOS-NO-cGMP-PKG system. This cascade may lead to cardiodepression. However, by using a dose of hrSTA-CgA1-78 that has a transient cardiodepressive effect, it is possible to trigger a preconditioning-like effect that involves A1 receptors, NOS, and PKC. Data suggest that the protective effects of hrSTA-CgA1-78 are mainly due to an adenosine-independent pathway (i.e., NOS-NO-cGMP-PKG pathway) and that only part of that protection may be due to an adenosine-dependent pathway (i.e., adenosine receptor-dependent PKC activation). Nevertheless, both pathways may converge on eNOS and PKC. In fact, the blockade of either eNOS or PKC prevented the infarct-sparing effect of hrSTA-CgA1-78, whereas A1 receptor blockade only partially inhibited this protective effect. Our data emphasize the potential importance of the release of CGA as an attempt of the cardiovascular system to protect itself against I/R damages and, eventually, against sympathetic overstimulation.

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