Glycosylated chromogranin A in heart failure - implications for processing and cardiomyocyte calcium homeostasis

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ABSTRACT

Background: Chromogranin A (CgA) levels have previously been found to predict mortality in heart failure (HF), but currently no information is available regarding CgA processing in HF and whether the CgA fragment catestatin (CST) may directly influence cardiomyocyte function.

Methods and Results: CgA processing was characterized in post-infarction HF mice and patients with acute HF and the functional role of CST was explored in experimental models. Myocardial biopsies from HF, but not sham-operated mice, demonstrated high molecular weight (hmw) CgA bands. Deglycosylation treatment attenuated hmw bands, induced a mobility shift, and increased shorter CgA fragments. Adjusting for established risk indices and biomarkers, circulating CgA levels were associated with mortality in patients with acute HF, but not in patients with acute exacerbation of chronic pulmonary obstructive disease. Low CgA to CST conversion was also associated with increased mortality in acute HF, thus supporting functional relevance of impaired CgA processing in CVD. CST was identified as a direct inhibitor of Ca^{2+}/calmodulin (CaM)-dependent protein kinase II δ (CaMKIIδ) activity, and CST reduced CaMKIIδ-dependent phosphorylation of phospholamban and the ryanodine receptor 2. In line with CaMKIIδ inhibition, CST reduced Ca^{2+} spark and wave frequency, reduced Ca^{2+} spark dimensions, increased sarcoplasmic reticulum Ca^{2+} content, and augmented the magnitude and kinetics of cardiomyocyte Ca^{2+} transients and contractions.

Conclusions: CgA to CST conversion in HF is impaired due to hyperglycosylation, which is associated with clinical outcomes in acute HF. The mechanism for increased mortality may be dysregulated cardiomyocyte Ca^{2+} handling due to reduced CaMKIIδ inhibition.

KEY WORDS: Chromogranin A; catestatin; biomarker; calcium cycling/excitation-contraction coupling; Ca^{2+} / calmodulin (CaM)-dependent protein kinase II

INTRODUCTION

Paracrine factors and hormones are important biomarkers and key targets for therapy in heart failure (HF). Accordingly, testing novel hormonal substances as biomarkers and potential therapeutic strategies may prove valuable to advance our understanding of the pathophysiology of HF.

Chromogranin A (CgA) is a 48 kDa pro-hormone that is produced in many tissues throughout the body, including in neuroendocrine and myocardial cells.[1-3] Previous studies have found circulating CgA levels to be associated with prognosis in patients with acute coronary syndromes[4-6], HF [7-9], and severe sepsis.[10] Furthermore, the association between CgA levels and clinical outcome has also been demonstrated in multivariable models that have adjusted for other risk variables.[4-6, 8-10] Thus, CgA appears to reflect pathophysiology not measured by the established risk indices in cardiovascular disease (CVD). Although originally considered a surrogate marker of adrenergic tone,[11] previous studies in patients with CVD have not found significant correlations between CgA and catecholamine levels.[5, 6, 8] In contrast, the literature supports that CgA influences myocardial function via the production of short cleavage fragments. The fragment catestatin (CST, CgA352-372) may be of particular relevance for CVD. CST has been found to reduce myocardial β-adrenergic and endothelin-1 signaling, to affect myocardial contractility, to attenuate myocardial ischemia-reperfusion injury,[12] and to have a direct effect on Ca^{2+} handling by interaction with calmodulin (CaM) in non-cardiac cells.[13] However, currently no information is available in the literature concerning the processing of CgA to shorter peptide fragments in HF. In addition, it is not established whether CgA fragments like CST may directly influence cardiomyocyte function. While previous studies have reported that CST modulates cardiomyocyte Ca^{2+} handling only via indirect mechanisms,[14] this finding is surprising given the direct effect of CST on Ca^{2+}

handling in non-cardiac cells,[13] the influence by other chromogranin-secretogranin proteins on cardiomyocyte Ca^{2+} handling,[15] and the ability of CST to directly interact with calmodulin (CaM).[13] CaM is an upstream activator of Ca^{2+}/ CaM-dependent protein kinase II δ (CaMKIIδ), which is an established regulator of cardiomyocyte Ca^{2+} handling.[16] Since overactive myocardial CaMKIIδ signaling constitutes a central mechanism of HF, direct regulation of this pathway by CST would link CgA and CST directly to CVD. Accordingly, the aims of this study were to characterize CgA processing in acute HF and to explore whether CST directly modulates cardiomyocyte Ca^{2+} handling via CaMKIIδ inhibition.

METHODS

Details can be found in the Online Appendix. Animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The clinical study was performed according to the Declaration of Helsinki, approved by the Regional Ethics Committees, and all participants provided written informed consent before study commencement.

Post-infarction Heart Failure Mouse Model and Immunoblotting

Experimental myocardial infarctions were induced by permanent ligation of the main left coronary artery, and the presence of HF was verified by echocardiography 1 week later. [3, 17] Biopsies from the infarcted and non-infarcted LV, right ventricle, and other organs were collected. Deglycosylation was performed by adding different combinations of enzyme cocktails. Short CST fragments were assessed by using a high percentage gel and subjecting the lower part of the membrane to longer exposure.

Clinical Study

We included 314 patients who were hospitalized due to acute dyspnea at Akershus University Hospital, Lørenskog, Norway into the ACE 2 Study (Supplemental Fig. 1). Patients were characterized as acute HF and acute exacerbation of chronic obstructive pulmonary disease (COPD) by an adjudication committee of two experts working independently.[18] CgA levels were measured by ELISA and CST levels by an in-house radioimmunoassay.[19] We calculated the percentage of CgA not converted to CST (CgA to CST conversion) in individual patients by this formula: ([CgA-CST]/CgA) x 100. High-sensitivity troponin T (hs-TnT), N-terminal pro-B-type natriuretic peptide (NT-proBNP), and norepinephrine (NE) levels were measured as previously reported.[15]

Effect by CST on Ca^{2+} Handling

CST-CaMKIIδ interactions were examined by immunoprecipitation, pull-down experiments, and by surface plasmon resonance. CaMKIIδ activity was assessed by *in vitro* kinase activity assays. Effects of CST on CaMKIIδ, ryanodine receptor 2 (RyR2), and phospholamban (PLB) phosphorylation were studied in Langendorff-perfused hearts. BayK was included in the perfusate to promote L-type Ca^{2+} channel opening and thereby increase CaMKIIδ activity. Adult ventricular cardiomyocytes were isolated as previously reported.[15] Contractions and Ca^{2+} transients were elicited by field stimulation, Ca^{2+} sparks were measured in resting cells, and cytosolic [Ca^{2+}] was detected by confocal microscopy and whole-cell photometry. Cells were pre-incubated and perfused with 45 nmol/L or 4.5 μmol/L CST.

Statistics

Clinical data are presented as mean ± SD and median (quartile [Q] 1-3) for biomarkers due to non-normal distributions, and experimental data as mean ± SEM. Differences between groups

were examined by Student´s *t*-test or the Mann-Whitney *U* test and the Chi-square test and correlation coefficients were calculated by the Spearman rank test. Data with multiple groups were assessed by the Kruskal-Wallis test. We present patients stratified according to biomarker quartiles in Kaplan-Meier plots and the association with mortality was examined by the log-rank test. Variables associated with mortality in the acute HF patients were also examined by single variable and multivariable Cox proportional hazard regression analysis with hazard ratios (HR) presented with 95% confidence intervals (CI). Variables associated with mortality in single variable analysis were included in the multivariable model (forward selection of variables). *P*-values <0.05 were considered significant.

RESULTS

Chromogranin A is Hyperglycosylated in the Failing Myocardium

Immunoblotting demonstrated high molecular weight (hmw) CgA bands in myocardial biopsies of HF, but not sham-operated animals (Fig. 1A). Short CgA fragments were observed in sham-operated animals, but to a much lesser degree in HF. The hmw CgA bands were also observed in the right ventricle in HF, but not other organs examined, neither in HF nor sham-operated animals (Supplemental Fig. 1). The short CST-fragment was also found to be highly reduced in LV of HF animals compared to CST levels in the LV of sham-operated animals (Fig. 1B). Adding enzymes that cleaved N- or O-linked glycosylation modifications yielded a shift in the myocardial hmw CgA bands (Fig. 1C). Combinations of enzymes that cleave O-linked glycosylation also reduced the appearance of hmw CgA bands, and yielded an increase in short CgA fragments. The increase in short CgA fragments was most prominent when all enzymes where added in combination (Fig. 1C, right lane).

Circulating CgA Levels Predict Mortality in Acute HF and Reduced CgA to CST Conversion is Associated with a Poor Outcome

In total, 314 patients were included in the ACE 2 Study and the final diagnosis was acute HF in 143 patients (46%) and acute exacerbation of COPD in 84 patients (27%), while 87 patients (28%) were hospitalized due to non-HF, non-COPD related dyspnea (Supplemental Fig. 2). Among the patients classified as hospitalized for acute HF, 51 patients (36%) were considered to have HF with preserved left ventricular ejection function (Supplemental Table 1). Admission CgA levels, but not CST levels, were higher in the acute HF patients compared to the patients with non-HF related dyspnea (Supplemental Table 1). The proportion of CgA molecules not converted to CST molecules was also higher in acute HF patients than in the patients with non-HF dyspnea: median 57% vs. median 41%, respectively, p=0.001 (Supplemental Table 1). Baseline CgA levels in the acute HF patients were inversely correlated with estimated creatinine clearance and diastolic blood pressure on admission, positively correlated with hs-TnT and NT-proBNP levels, and not significantly correlated with NE levels (Supplemental Table 2). CST levels were inversely correlated with age, but not correlated to other clinical or laboratory variables (Supplemental Table 2).

During median follow-up of 817 days, 66 patients (46%) with acute HF died, 35 patients (42%) with acute COPD died, and 13 patients (15%) with non-HF, non-COPD related dyspnea died (Supplemental Fig. 2). To include relatively homogenous patient categories with similar mortality rates during follow-up, we selected the patients hospitalized with HF or COPD for further studies. Patients with the highest CgA levels on admission for acute HF had a significantly higher risk of mortality during follow-up compared to the other HF patients (Fig. 2A; p=0.001 by the log-rank test). Within 200 days of follow-up, 50% of HF patients with 4^{th} quartile CgA levels had died compared to <10% of HF patients with CgA levels in

the 1st quartile. CgA was also found to be a strong prognostic marker in Cox regression analysis, including in multivariable analysis that included NT-proBNP, hs-TnT, and NE levels: HR [$_{ln}$CgA per 1 standard deviation increase] 1.51 (95% CI 1.16-1.95), p=0.002 (Table). In contrast, baseline CST levels did not predict mortality in patients hospitalized with acute HF (Table and Fig. 2B). Pertinent to this point, patients with low CgA to CST conversion had a worse outcome compared to the other HF patients (Fig. 2C). CgA levels, CST levels, and CgA to CST conversion were not associated with mortality in the patients hospitalized with acute exacerbation of COPD (Supplemental Fig. 3).

CST Interacts Directly with CAMKIIδ and Reduces CAMKIIδ Activity

As we found low CgA to CST conversion to be associated with mortality in acute HF, but not in acute exacerbation of COPD, we aimed to explore whether CST may directly affect cardiomyocyte function. The short CgA fragment CST shows sequence similarity to the autoregulatory domain of CaMKIIδ containing the CaM binding site, which suggests that CST might bind directly to the substrate binding site in the catalytic domain of CaMKIIδ (Fig. 3A). As predicted by bioinformatics, we identified a CST-CaMKIIδ interaction by immunoprecipitation (Fig. 3B) and pull-down experiments (Fig. 3C) using recombinant His-CaMKIIδ (T287D) and biotin-CST. The direct interaction between CST and CaMKIIδ was also demonstrated by surface plasmon resonance analyses with a K_D = (5±3) x 10^{-7} M, a k_a = (2±1) x 10^3 $M^{-1}s^{-1}$, and a k_d = (8±3) x $10^{-4}s^{-1}$ (Fig. 3D).

Based on the interaction between CST and CaMKIIδ we investigated if CST directly affects CaMKIIδ activity. CaMKIIδ can be autophosphorylated at Thr286 in the regulatory domain of CaMKIIδ, which leads to prolonged kinase activity.[16] We observed that CST reduced the

autophosphorylation in BayK-perfused mouse hearts (Fig. 3E), while CST did not reduce the basal level of autophosphorylation (Supplemental Fig. 4A). CST was also observed to strongly inhibit CaMKIIδ activity in a dose-dependent manner in an *in vitro* kinase assay (Fig. 3F).

CST Reduces CaMKIIδ-Dependent Phosphorylation of the Ryanodine Receptor 2 and Phospholamban

CaMKIIδ is known to phosphorylate proteins involved in cardiomyocyte Ca^{2+} homeostasis and we examined whether CST could influence RyR2 phosphorylation and SERCA via phosphorylation of PLB in isolated perfused mouse hearts. CST reduced both the basal level and the BayK-induced increase in Ser2814-RyR2 phosphorylation (Fig. 4A, Supplemental Fig 4B), which is regulated by CaMKIIδ.[16] This strong inhibitory effect was not observed at the Ser2808-RyR2 phosphorylation site (Fig. 4B, Supplemental Fig 4C), which is regulated by protein kinase A (PKA).[21] We also found that CST reduced the basal level and the BayK-induced increase in phosphorylation of PLB at Thr17-PLB (Fig. 4C, Supplemental Fig 4D) and reduced the BayK-induced increase in phosphorylation of PLB at Ser16-PLB but not the basal level (Fig. 4D, Supplemental Fig. 4E), which are regulated by CaMKIIδ and PKA, respectively.[16]

CST Modulates Cardiomyocyte Ca^{2+} Homeostasis

We next examined effects of CST on Ca^{2+} handling in isolated cardiomyocytes. As expected of a CaMKIIδ inhibitor,[22] we observed that CST (4.5 μM) prominently reduced the dimension of Ca^{2+}sparks including the full duration at half-maximum, full width at half-maximum intensity, and the time to peak of the Ca^{2+}spark (Fig. 5A). Ca^{2+}spark frequency and Ca^{2+} wave

frequency were also markedly reduced (Fig. 5A). A similar reduction in Ca^{2+} spark dimensions and the frequency of Ca^{2+} sparks and waves was observed when the experiment was repeated with a 100-fold lower CST concentration (45 nmol/L, Fig. 5B), which is in the range of circulating CgA levels in the HF patients. Ca^{2+} transients and contractions were larger across a range of stimulation frequencies (1, 0.5, 2 and 5 Hz) (Fig. 6A, B). Kinetics of both contraction and relaxation were more rapid in CST-treated cells than controls (Fig. 6A), an effect likely explained by faster declining Ca^{2+} transients (Fig. 6B). Frequency-dependent acceleration of relaxation (FDAR) and Ca^{2+} transient decline was significantly attenuated in the CST treatment group (Fig. 6A, B). Consistent with reduced sarcoplasmic reticulum (SR) Ca^{2+} leak (sparks, Fig. 5), we observed that CST treatment increased SR Ca^{2+} content (Fig. 6C) without altering rates of Ca^{2+} extrusion from the cell or Ca^{2+} reuptake into the SR (Supplemental Fig. 5).

DISCUSSION

The main results of this study are (1) that CgA is hyperglycosylated in the failing myocardium, (2) that the ratio between circulating CgA-to-CST levels, but not CST levels alone, provides prognostic information in patients with acute HF, and (3) that CST has cardioprotective effects via direct CaMKIIδ inhibition. Accordingly, as CaMKII activity is increased in HF,[16] myocardial CgA hyperglycosylation with reduced CgA to CST processing should be detrimental due to impaired local CaMKII control. Indeed, we found that acute HF patients with low CgA to CST conversion had a worse outcome compared to HF patients with higher CgA to CST conversion.

CgA has also previously been recognized as a biomarker that provides prognostic information in patients with CVD. Earlier studies have examined CgA in patients with ACS[4-6], HF [7-9], and severe sepsis,[10] and in agreement with our present data, these studies found CgA to provide additional prognostic information to established risk indices, including biomarkers and left ventricular function.[4-6, 8-10] The complementary information provided by CgA is also reflected by only modest correlations between CgA and BNP or NT-proBNP levels,[4, 6, 7, 9, 10] and no correlation between CgA and catecholamine levels in patients with CVD.[5, 6] We now advance the present knowledge regarding CgA in CVD by demonstrating CgA hyperglycosylation in the failing myocardium and that this may have direct functional consequences via reduced CST-mediated CaMKII inhibition. Based on knowledge from other proteins, myocardial glycosylation is increased in the failing myocardium and represents a mechanism for post-translational protein modification. Pertinent to this point, processing of glycosylated proteins can be impaired by sugar groups blocking the binding of proteases to cleavage sites. A prominent example of this is pro-BNP, which is extensively glycosylated in the middle and N-terminal end of the molecule,[18] and where processing to BNP and NT-proBNP is impaired by the glycosylation. As CgA processing takes place at several dibasic cleavage sites along the full-length molecule,[2] extensive CgA glycosylation could influence CgA processing and our results support this model as short CgA and CST fragments are increased after exposing myocardial biopsies to deglycosylation enzymes. Hence, a substantial proportion of CgA molecules, at least in the failing myocardium, could be glycosylated and thus inaccessible for further processing. Additional mechanism for CST removal may be degradation pathways of CgA to fragments other than CST or factors that independently affect the breakdown or stability of CST, which both will have to be assessed in additional studies.

Although no group previously has reported cardiac-specific glycosylation of CgA, we believe our results are supported by the literature. A previous report has found circulating CST levels to be reduced in patients with subclinical and established HF compared to subjects free from HF,[23] which supports reduced CgA to CST processing as CgA levels are known to be increased in HF patients[8] (as also demonstrated in our clinical cohort). Another group has also reported impaired CgA to CST processing in myocardial biopsies from aging mice.[24] However, these authors did not report immunoblot bands above 100 kDa and thus whether the reduction in CST was paralleled by increments in hmw CgA bands is unknown. Still, this study[24] and another more recent study[25] both observed CgA immunoblot bands above the expected full-length molecular weight of 74 kDa in myocardial tissue samples. In contrast, no such bands were evident in adrenal gland tissue samples. These groups also suggested that the myocardial hmw CgA bands could be due to glycosylation, but they did not follow-up on this hypothesis. Finally, using HPLC to characterize plasma CgA fragments in stable HF patients, Pieroni et al also found peaks with molecular weights above the peak considered representative for full-length-CgA,[1] which could represent the release of glycosylated CgA molecules into the circulation.

We found reduced CgA to CST conversion to be associated with a poor outcome in acute HF, but not in patients with acute exacerbation of COPD, which supports a role for CST in cardiomyocyte pathophysiology. Based on our data of CST as a CaMKII inhibitor and previous data that found CST to reduce myocardial ischemia-reperfusion injury,[12] increase cardiomyocyte cell survival,[26] and attenuate myocardial β-adrenergic and endothelin-1 signaling,[27] CST seems to be a counter-regulatory paracrine ligand of relevance in CVD. Given the paramount importance of hyperactive CaMKIIδ signaling in CVD,[16] the primary role of CST may be as part of a paracrine/autocrine protective feedback loop that could

antagonize overactive myocardial CaMKIIδ signaling. However, our data also indicate that this system may be malfunctioning in a proportion of HF individuals due to myocardial CgA hyperglycosylation. Accordingly, although we previously have reported increased CgA mRNA levels[4] and increased prohormone convertases (PC) 1/3 and PC2 activity in the failing myocardium,[17] which are the principal proteases for CgA cleavage,[28] CgA glycosylation could block the binding of these proteases to the full-length molecule and thus reduce CST production. Moreover, although a recent report found isoprenalin- and endothelin-infusion to increase myocardial CgA processing in healthy hearts,[25] whether the same increased processing would occur in failing hearts with extensive CgA glycosylation is unknown.

CaMKIIδ is a nodal kinase in the regulation of cardiomyocyte Ca^{2+} handling.[16] CaMKIIδ is known to be hyperactive in several cardiac pathologies, including HF, ischemia, remodeling, cardiomyocyte cell death, and arrhythmias.[16] Hence, CaMKIIδ inhibition is recognized as an interesting target for attenuation of disease progression.[16, 29] CST exhibits the CaM binding motif 1-5-10 and shows homology to the sequence surrounding the core pseudosubstrate site (RKLK) in CaMKIIδ,[20] indicating that CST could also bind to CaMKIIδ. The ability of CST to interact directly with CaMKIIδ is consistent with prior work demonstrating that CST and other granin-derived fragments can bind to CaM in non-cardiac cells[13] and to CaM and CaMKIIδ in cardiomyocytes.[15] Our data on Ca^{2+} handling in the presence of CST are also consistent with CST acting as CaMKIIδ inhibitor since CaMKIIδ inhibition has been found to reduce Ca^{2+} spark frequency and magnitude, augment sarcoplasmic reticulum Ca^{2+} content, and increase the magnitude of Ca^{2+} transients.[22, 30] As expected with CaMKIIδ inhibition, faster transient decay during CST treatment was also associated with more rapid contraction kinetics.[31] CST also blunted FDAR and Ca^{2+} transients, a phenomenon reported to result from

activation of SERCA at high frequencies, following CaMKIIδ-dependent phosphorylation of phospholamban at Thr17.[32] The observed effects of CST on cardiomyocyte Ca^{2+} handling are in agreement with the effects of other CaMKIIδ-inhibitors like KN-93 and AIP[32] and our recent report on the granin protein secretoneurin.[15] Hence, a possible integrated model could be that (1) the most severely ill HF patients increase CgA production as a compensatory mechanism, (2) myocardial hyperglycosylation reduces the effect of this paracrine system by blocking CgA to CST conversion, and (3) reduced CST production is detrimental due to lack of local CaMKIIδ control. Based on this model, targeting myocardial CgA hyperglycosylation to increase local CST production could prove valuable as a future strategy to improve outcome in HF. Still, we acknowledge that additional work is needed to decipher the mechanism whereby the glycosylation occurs and whether interventions to reduce CgA glycosylation would lead to increased myocardial CST levels in subjects with HF. Pertinent to this point; we also found CST to reduce Ser16-PLB phosphorylation, which is not recognized as a CaMKII phosphorylation site. Accordingly, CST could have effects also outside of CaMKII inhibition, including effects via the NO-cGMP pathway, analogously to data previously demonstrated,[27, 33] or CST could compete with PLB for phosphorylation. Thus, there is a need for experimental studies to establish additional processes that CST could regulate in the failing myocardium, besides CaMKII inhibition, which was the focus of the current work.

In conclusion, we found CgA-to-CST ratio, but not CST alone, to be a valuable prognostic biomarker in acute HF. Moreover, we demonstrate increased myocardial CgA glycosylation and impaired CgA processing in HF, which should be considered detrimental as CST reduces diastolic Ca^{2+} leak via direct CaMKIIδ inhibition. Thus, although CgA production seems to

increase and probably represents a counter-regulatory mechanism in HF, this system may malfunction due to augmented myocardial CgA glycosylation.

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CONFLICT OF INTEREST

No disclosures.

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FIGURE LEGENDS

Figure 1. Post-translational modifications and processing of CgA in HF. (A) Immunoblotting demonstrates hmw CgA bands in myocardial biopsies of HF, both from the viable area and from the infarcted area. No hmw bands are observed in sham-operated animals. Short CgA fragments including CST were observed in sham-operated animals, but to a much lesser degree in HF (sham: n=15; HF (viable): n=16; HF (infarct): n=6). **(B)** By immunoblotting the short CST-fragment was found to be highly reduced in HF animals compared to in sham-operated animals (n=2). **(C)** Adding enzymes that cleaved N- or O-linked glycosylation modifications yielded a shift in the myocardial hmw CgA bands. Some combination of enzymes also reduced the appearance of hmw CgA bands, and yielded an increase in short CgA fragments (n=6). The increase in short fragments was most prominent when all enzymes where added.

Figure 2. Associations between CgA levels, CST levels, and CgA to CST conversion in patients with acute HF. Stratifying patients according to admission **(A)** CgA quartiles, but not **(B)** CST quartiles, provided excellent risk stratification in the patients with acute HF. **(C)** Patients with low CgA to CST conversion had worse outcome compared to the other HF patients (n=143).

Figure 3. CST is an endogenous CaMKIIδ inhibitor via direct binding. (A) CST from different species exhibits sequence similarities to the CaM binding region in CaMKIIδ; the CaM binding motif 1-5-10 is conserved in all species shown except mouse (red box). Black boxes indicate identical or functionally similar amino acids (DNA Star, Lasergene). RKLK: Stars denotes the core pseudosubstrate site in CaMKIIδ (RKLK).[20] CST-CaMKIIδ interaction

was demonstrated by **(B)** immunoprecipitation (n=4) and **(C)** pull-down experiments using recombinant His-CaMKIIδ (T287D) (active) and biotin-CST (n=4). **(D)** The direct binding of CST-CaMKIIδ was verified by surface plasmon resonance analysis (n=5). The binding response (grey) was overlaid with the fit of a 1:1 interaction model (black). **(E)** CST reduced autophosphorylation of Thr286-CaMKII in BayK-perfused hearts (BayK: n=5; CST+BayK: n=4). *p≤ 0.05, examined by Student´s t-test. **(F)** CaMKII activity was decreased by CST in a dose-dependent manner measured by an *in vitro* kinase assay (n=6-10). CN21a: positive control. Negative results are due to inhibition of autophosphorylation of CaMKIIδ. ****p≤0.001, examined by ANOVA and kruskal-wallis test**.**

Figure 4. CST reduced CaMKIIδ-dependent phosphorylation of the ryanodine receptor and phospholamban. (A) CST reduced BayK-induced Ser2814-RyR2 phosphorylation, **(B)** but not Ser2808-RyR2 phosphorylation. **(C)** CST reduced BayK-induced CaMKII-dependent phosphorylation of Thr17-PLB, and **(D)** BayK-induced Ser16-PLB phosphorylation. (BayK: n=5; CST+BayK: n=4) *p≤ 0.05, ***p≤ 0.001, examined by Student´s t-test.

Figure 5. CST reduced diastolic Ca^{2+} leak. CST reduced Ca^{2+} sparks in all dimensions and reduced Ca^{2+} spark and wave frequency using either **(A)** 45 nmol/L or **(B)** 4.5 μmol/L of CST. (Ca^{2+} sparks: Ctr: n_{hearts}=3, n_{cells}=12; CST;4.5 μM: n_{hearts}=3, n_{cells}=9, Ca^{2+} sparks: Ctr: n_{hearts}=9, n_{cells}=49; CST; 45 nM: $n_{hearts=}$8, n_{cells}=25) *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001, eamined by Student´s t-test.

Figure 6. CST augmented cardiomyocyte calcium transients and contractions. (A) CST treatment induced larger and faster contractions of cardiomyocytes across a range of stimulation frequencies (1, 0.5, 2 and 5 Hz). ***p≤ 0.001, examined by ANOVA and kruskal-

wallis test**.** **(B)** CST increased the magnitude of cardiomyocyte Ca^{2+} transients, and reduced the half relaxation time at all frequencies. ***p≤ 0.001, examined by ANOVA and kruskal-wallis test**. (C)** SR Ca^{2+} content was increased by CST. (Contractions: Ctr: n_{hearts}=3, n_{cells}=5-12; CST: n_{hearts}=2, n_{cells}=5-8; Ca^{2+} transients: Ctr: n_{hearts}=3, n_{cells}=13; CST: n_{hearts}=4, n_{cells}=7; SR content, 10 mM caffeine: Ctr: n_{hearts}=5, n_{cells}=17; CST: n_{hearts}=4, n_{cells}=15) *$p\leq 0.05$, examined by Student´s *t*-test.

Table. Predictors of mortality during follow-up in patients with acute HF.

Single variable analysis

	Hazard ratio	95 % CI	p
Age, per 10 year increase	1.50	1.17-1.92	0.001
Male gender	0.53	0.32-0.86	0.010
Body mass index, per 1 unit increase	0.94	0.89-0.99	0.012
Creatinine clearance, per 1 SD[*] increase (mL/min)	0.49	0.33-0.72	<0.001
Heart rate, admission, per 10 b.p.m increase	0.94	0.85-1.05	0.27
Systolic blood pressure, admission, per 10 mmHg increase	0.89	0.82-0.97	0.006
Diastolic blood pressure, admission, per 10 mmHg increase	0.76	0.65-0.90	0.001
NYHA[†] functional class IV vs. II/III	2.01	1.23-3.29	0.006
LVEF[‡], per 10% increase	1.00	0.83-1.20	0.99
History of			
Heart failure	1.43	0.86-2.38	0.17
Myocardial infarction	1.00	0.62-1.63	1.00
PCI[§]	0.69	0.37-1.29	0.24
CABG[‖]	1.17	0.66-2.05	0.59
Hypertension	0.83	0.51-1.36	0.47
Atrial fibrillation	1.07	0.66-1.74	0.78
Diabetes mellitus	1.78	1.08-2.95	0.024
COPD[#]	1.85	1.14-3.01	0.013

ln[**]CgA[††], admission, per 1 SD[*] increase	1.48	1.20-1.83	<0.001
ln[**]CST[‡‡], admission, per 1 SD[*] increase	1.02	0.81-1.29	0.86
ln[**]NT-proBNP[§§], admission, per 1 SD[*] increase	1.77	1.34-2.34	<0.001
ln[**]hs-TnT[‖‖] , admission, per 1 SD[*] increase	1.37	1.10-1.71	0.005
ln[**]NE[##], admission, per 1 SD[*] increase	1.45	1.13-1.85	0.004

Multivariable analysis: variables retained in the final model

	Hazard ratio	**95 % CI**	**p**
Age, per 10 year increase	1.57	1.17-2.10	0.002
Body mass index, per 1 unit increase	0.95	0.90-1.00	0.042
Diastolic blood pressure, admission, per 10 mmHg increase	0.82	0.70-0.96	0.014
Diabetes mellitus	2.15	1.24-3.73	0.007
History of COPD[#]	2.17	1.27-3.71	0.005
ln[**]CgA[††], admission, per 1 SD[*] increase	1.51	1.16-1.95	0.002
ln[**]NT-proBNP[§§], admission, per 1 SD[*] increase	1.57	1.13-2.18	0.007
ln[**]NE[##], admission, per 1 SD[*] increase	1.40	1.07-1.85	0.015

[*]SD indicates standard deviation; [†]NYHA=New York Heart Association; [‡]LVEF = left ventricular ejection fraction; [§]PCI [=] percutaneous coronary intervention; [‖]CABG = coronary artery bypass grafting; [#]COPD = chronic obstructive pulmonary disease; [**]ln = natural logarithm; [††]CgA= chromogranin A; [‡‡]CST= catestatin; [§§]NT-proBNP = N-terminal proBNP; [‖‖]hs-TnT = high-sensitivity cardiac troponin T; and [##]NE= norepinephrine

Figure 1

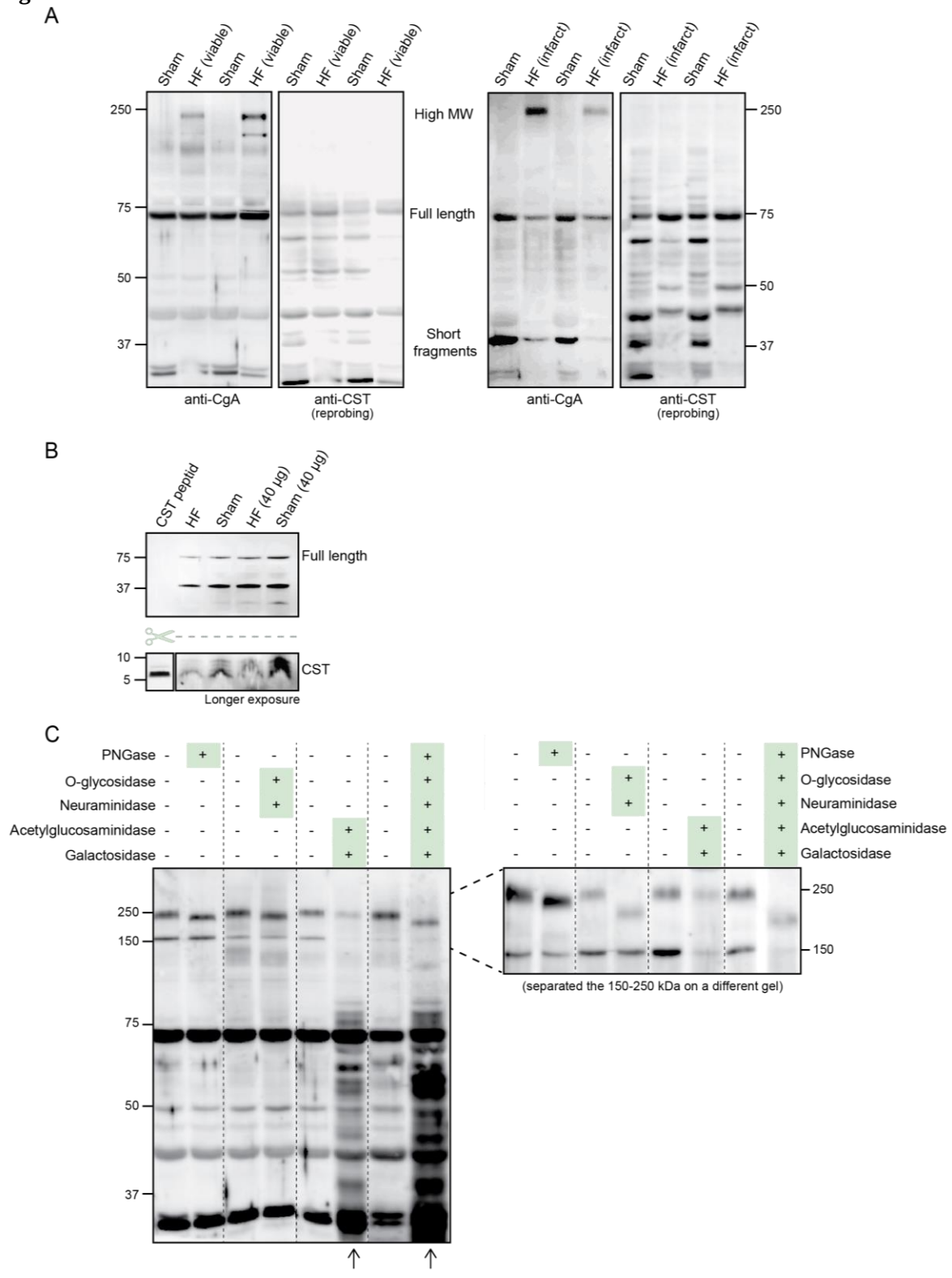

A
Sham
HF (viable)
Sham
HF (viable)
Sham
HF (viable)
Sham
HF (viable)
Sham
HF (infarct)
Sham
HF (infarct)
Sham
HF (infarct)
Sham
HF (infarct)
250
75
50
37
High MW
Full length
Short fragments
anti-CgA
anti-CST (reprobing)
anti-CgA
anti-CST (reprobing)
B
CST peptid
HF
Sham
HF (40 µg)
Sham (40 µg)
75
37
Full length
10
5
CST
Longer exposure
C
PNGase
O-glycosidase
Neuraminidase
Acetylglucosaminidase
Galactosidase
250
150
75
50
37
(separated the 150-250 kDa on a different gel)

Figure 2

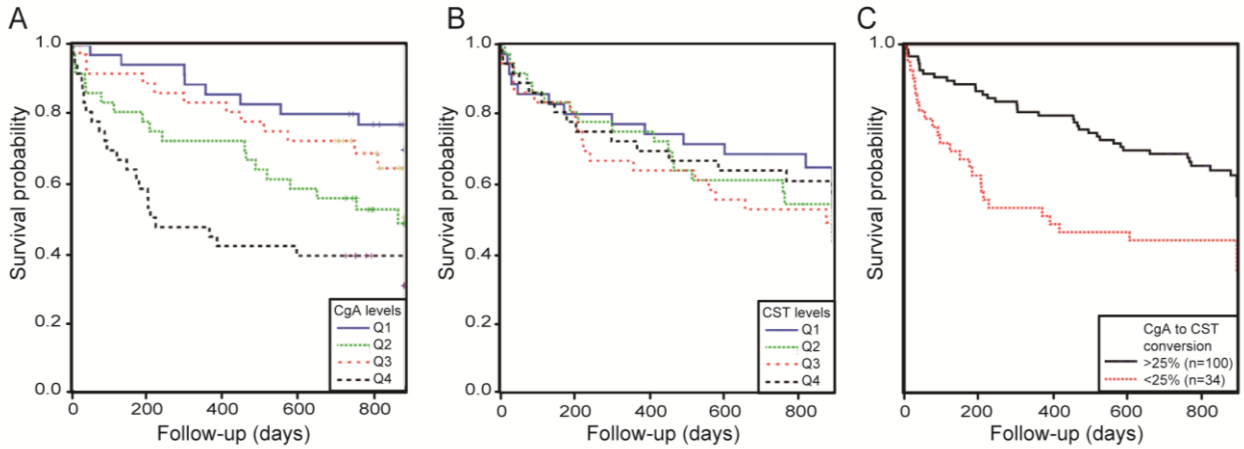

A
B
C
Survival probability
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
Follow-up (days)
CgA levels
Q1
Q2
Q3
Q4
CST levels
Q1
Q2
Q3
Q4
CgA to CST conversion
>25% (n=100)
<25% (n=34)

Figure 3

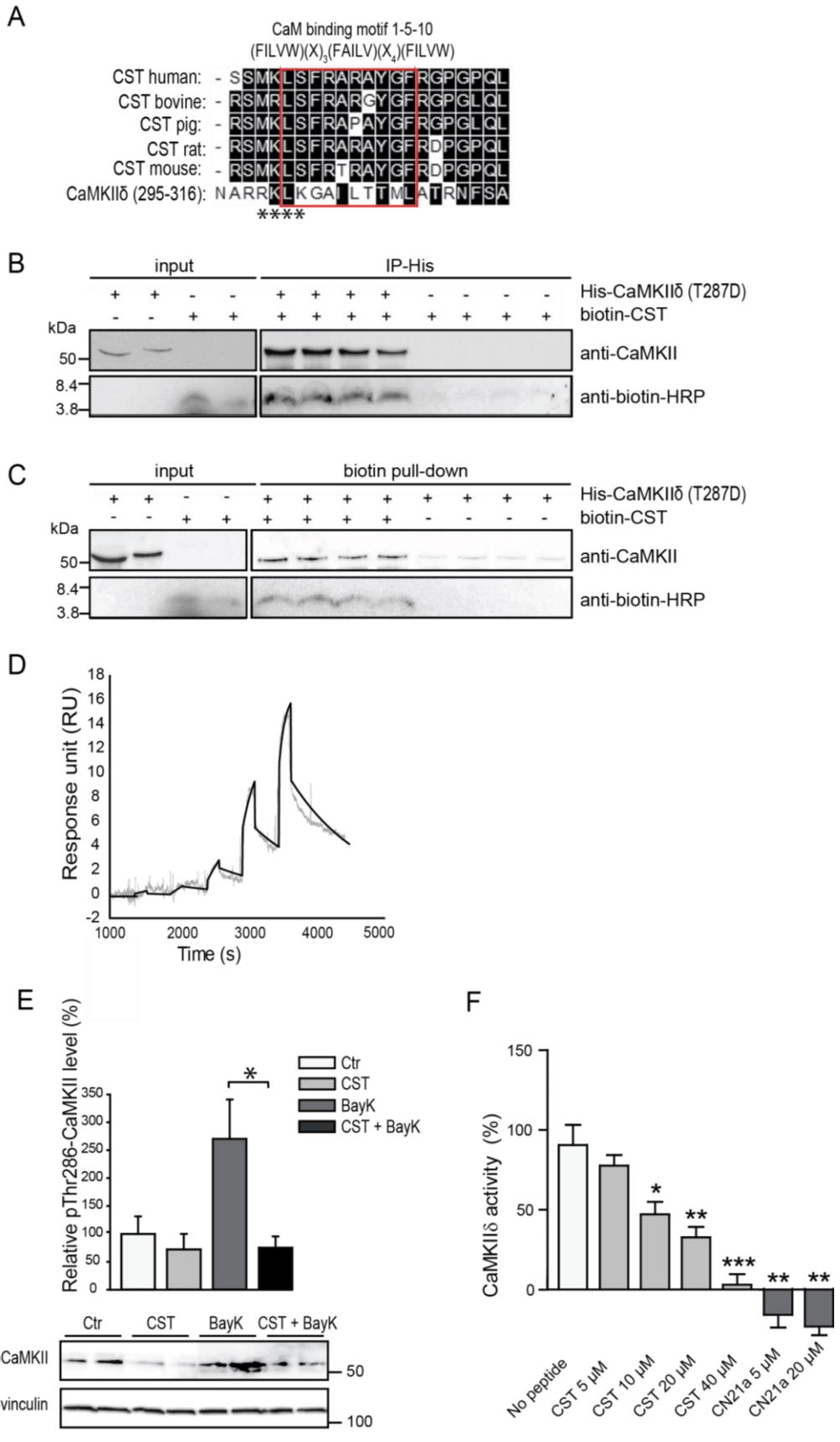

A
CaM binding motif 1-5-10
(FILVW)(X)3(FAILV)(X4)(FILVW)
CST human:
CST bovine:
CST pig:
CST rat:
CST mouse:
CaMKIIδ (295-316): NARRKLKGAILTTMLATRNFSA
****
B
input
IP-His
His-CaMKIIδ (T287D)
biotin-CST
kDa
50
8.4
3.8
anti-CaMKII
anti-biotin-HRP
C
input
biotin pull-down
His-CaMKIIδ (T287D)
biotin-CST
kDa
50
8.4
3.8
anti-CaMKII
anti-biotin-HRP
D
Response unit (RU)
Time (s)
E
Relative pThr286-CaMKII level (%)
Ctr
CST
BayK
CST + BayK
anti-pCaMKII
anti-vinculin
50
100
F
CaMKIIδ activity (%)
No peptide
CST 5 μM
CST 10 μM
CST 20 μM
CST 40 μM
CN21a 5 μM
CN21a 20 μM

Figure 4

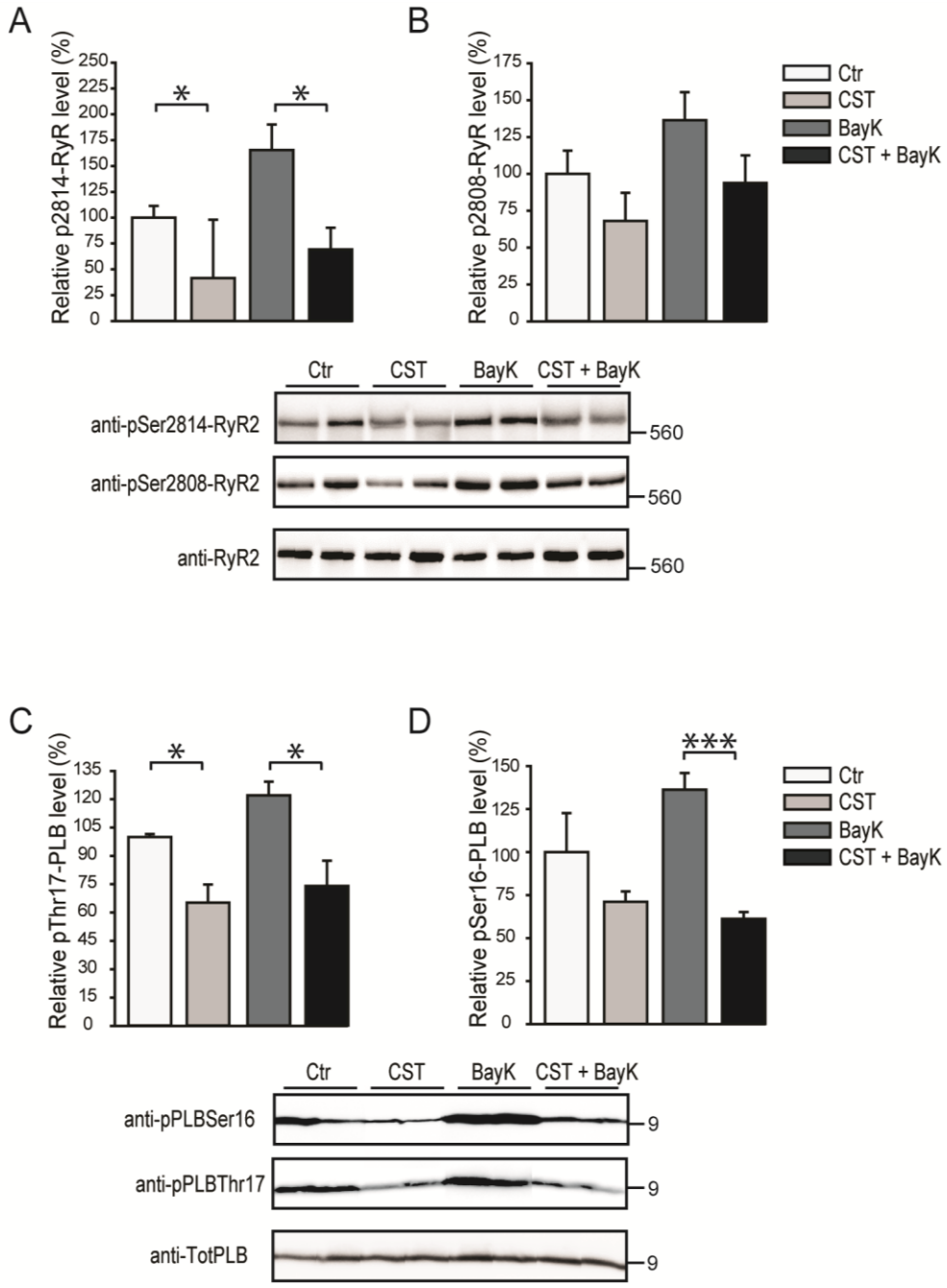

A
Relative p2814-RyR level (%)
250
225
200
175
150
125
100
75
50
25
0
*
*
B
Relative p2808-RyR level (%)
175
150
125
100
75
50
25
0
Ctr
CST
BayK
CST + BayK
Ctr
CST
BayK
CST + BayK
anti-pSer2814-RyR2
anti-pSer2808-RyR2
anti-RyR2
560
560
560
C
Relative pThr17-PLB level (%)
135
120
105
90
75
60
45
30
15
0
*
*
D
Relative pSer16-PLB level (%)
150
125
100
75
50
25
0
***
Ctr
CST
BayK
CST + BayK
Ctr
CST
BayK
CST + BayK
anti-pPLBSer16
anti-pPLBThr17
anti-TotPLB
9
9
9

Figure 5

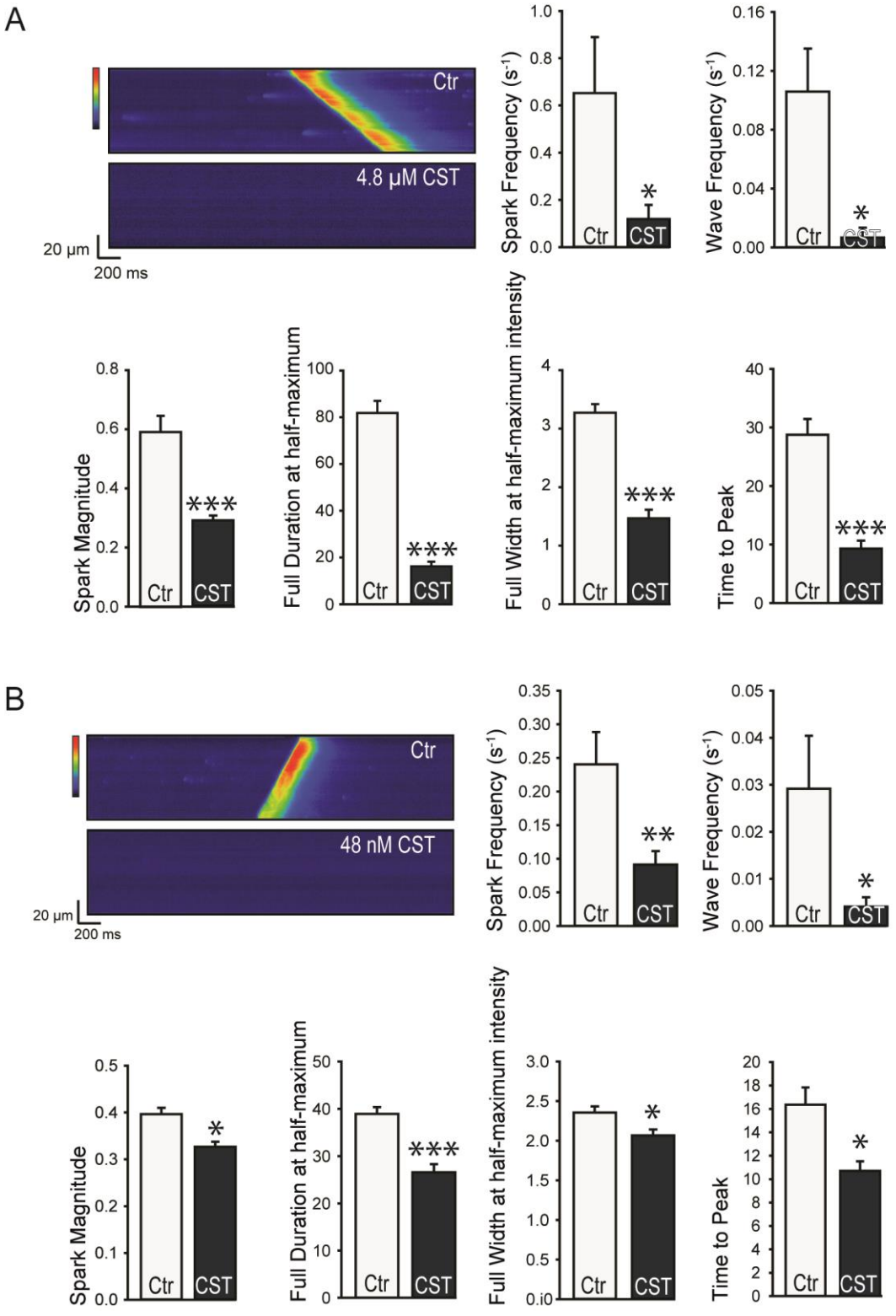
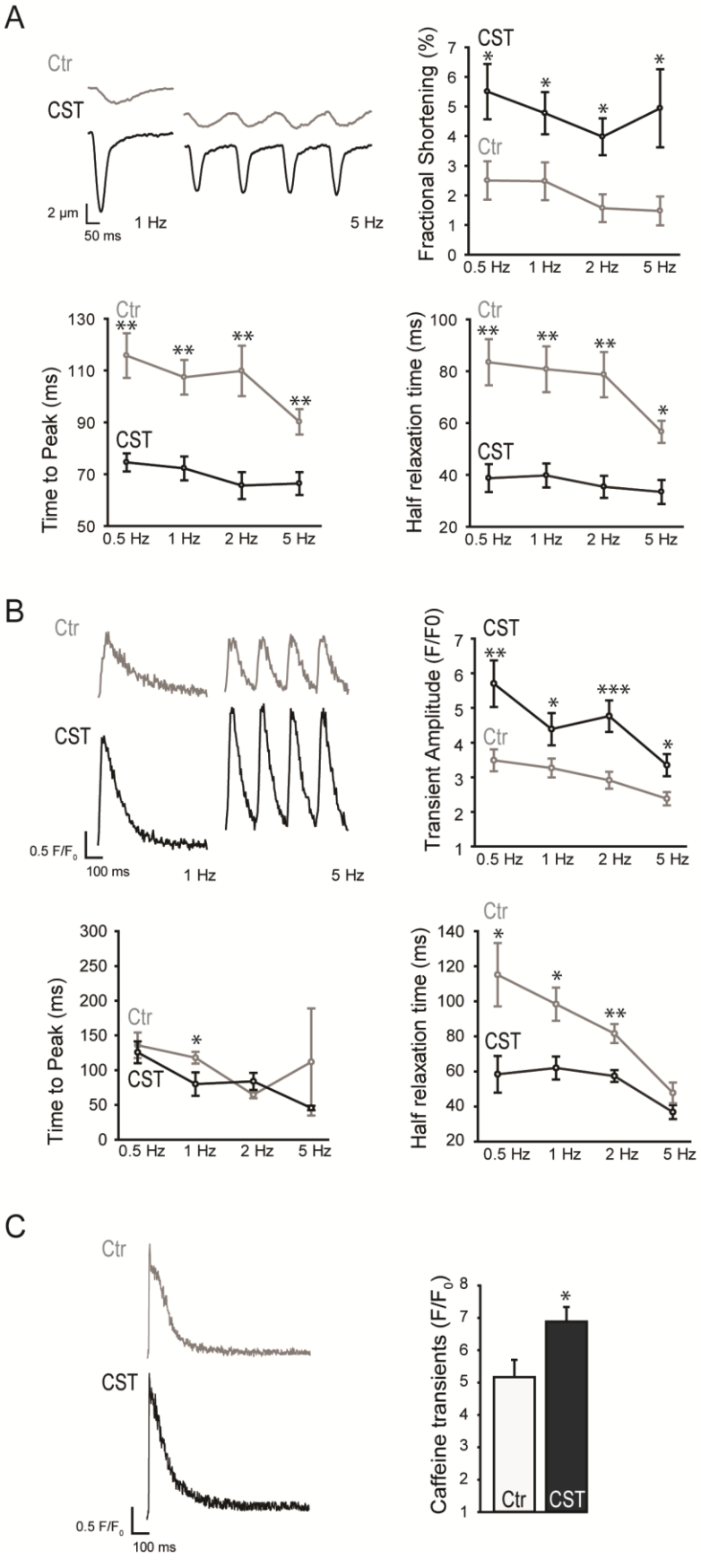

A
Ctr
4.8 µM CST
20 µm
200 ms
Spark Frequency (s-1)
Wave Frequency (s-1)
Spark Magnitude
Full Duration at half-maximum
Full Width at half-maximum intensity
Time to Peak
Ctr
CST
B
Ctr
48 nM CST
20 µm
200 ms
Spark Frequency (s-1)
Wave Frequency (s-1)
Spark Magnitude
Full Duration at half-maximum
Full Width at half-maximum intensity
Time to Peak
Ctr
CST

Figure 6


A
Ctr
CST
2 µm
50 ms
1 Hz
5 Hz
Fractional Shortening (%)
CST
Ctr
0.5 Hz
1 Hz
2 Hz
5 Hz
Time to Peak (ms)
Half relaxation time (ms)
B
Ctr
CST
0.5 F/F0
100 ms
1 Hz
5 Hz
Transient Amplitude (F/F0)
Time to Peak (ms)
Half relaxation time (ms)
C
Ctr
CST
0.5 F/F0
100 ms
Caffeine transients (F/F0)
Ctr
CST