Partial Inhibition of Sodium/Calcium Exchange Restores Cellular Calcium Handling in Canine Heart Failure

Ion A. Hobai, Christoph Maack, Brian O’Rourke

Abstract—Sodium/calcium (Na\(^+\)/Ca\(^{2+}\)) exchange (NCX) overexpression is common to human heart failure and heart failure in many animal models, but its specific contribution to the cellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) handling deficit is unclear. Here, we investigate the effects of exchange inhibitory peptide (XIP) on Ca\(^{2+}\) handling in myocytes isolated from canine tachycardia pacing-induced failing hearts. Whole-cell patch-clamped left ventricular myocytes from failing hearts (F) showed a 52% decrease in steady-state sarcoplasmic reticulum (SR) Ca\(^{2+}\) load and a 44% reduction in the amplitude of the [Ca\(^{2+}\)]\(_{i}\) transient, as compared with myocytes from normal hearts (N). Intracellular application of XIP (30 \(\mu\)mol/L) normalized the [Ca\(^{2+}\)]\(_{i}\) transient amplitude in F (3.86-fold increase), concomitant with a similar increase in SR Ca\(^{2+}\) load. The degree of NCX inhibition at this concentration of XIP was \(\approx 27\%\) and was selective for NCX: L-type Ca\(^{2+}\) currents and plasmalemmal Ca\(^{2+}\) pumps were not affected. XIP also indirectly improved the rate of [Ca\(^{2+}\)]\(_{i}\) removal at steady-state, secondary to Ca\(^{2+}\)-dependent activation of SR Ca\(^{2+}\) uptake. The findings indicate that in the failing heart cell, NCX inhibition can improve SR Ca\(^{2+}\) load by shifting the balance of Ca\(^{2+}\) fluxes away from trans-sarcolemmal efflux toward SR accumulation. Hence, inhibition of the Ca\(^{2+}\) efflux mode of the exchanger could potentially be an effective therapeutic strategy for improving contractility in congestive heart failure. (Circ Res. 2004;95:000-000.)

Key Words: exchange inhibitor peptide • XIP • excitation-contraction coupling • calcium transient

Altered calcium (Ca\(^{2+}\)) handling is a key factor in the pathophysiology of heart failure. A typical failing heart cell shows a decrease in the ability of the internal stores (the sarcoplasmic reticulum [SR]) to load with Ca\(^{2+}\), and an increase in Ca\(^{2+}\) extrusion from the cell by the sodium/calcium exchanger (NCX). NCX overexpression is a component of altered Ca\(^{2+}\) handling in human and animal models, \(^2,^3\) but it is unclear whether it is compensatory or contributes to dysfunction. One widely held hypothesis is that NCX overexpression compensates for decreased Ca\(^{2+}\) re-uptake into the SR by increasing Ca\(^{2+}\) extrusion from the cell, \(^4,^5\) which improves relaxation (positive lusitropic) but at the cost of a further depletion of SR Ca\(^{2+}\) stores (negative inotropic). Further complicating the issue is the observation that NCX overexpression is also found in hypercontractile models with no SR dysfunction. \(^6\)

We studied the effect of partially correcting the NCX overexpression (by applying an exchange inhibitory peptide [XIP]) in a canine model of heart failure. Partial inhibition of NCX normalized both SR Ca\(^{2+}\) release and re-uptake, arguing for a critical role for NCX overexpression in the Ca\(^{2+}\) handling deficit as well as for its potential as a therapeutic target.

Methods
These experiments were performed using a canine tachycardia pacing-induced heart failure model. We, \(^7,^8\) and others, \(^9\) have previously demonstrated that this animal model reproduces a remarkable number of features of the human disease. Induction of heart failure, isolation of midmyocardial cardiomyocytes, single-cell electrophysiology studies, and Ca\(^{2+}\) measurements were performed (at 37°C) as previously described, \(^2\) as summarized in the expanded Methods section in the online data supplement available at http://circres.ahajournals.org.

Excitation–Contraction Coupling Experiments
The main experimental protocol (Figures 1 through 5) consisted of a train of 0.5-second depolarizations from −80mV to +10mV, applied at 0.5 Hz until steady-state, followed by a rapid application of caffeine to measure SR Ca\(^{2+}\) load. The external solution contained (mmol/L): NaCl 140; KCl 1; CaCl\(_2\) 2; MgCl\(_2\) 1; HEPES 5; Glucose 10; niflumic acid 0.1 (to block Ca\(^{2+}\)-activated Cl\(^{−}\) currents), pH 7.4. After reaching steady-state, 30 \(\mu\)mol/L tetrodotoxin (Na\(^+\) channel blocker) was applied, to allow a better estimation of the peak of the L-type Ca\(^{2+}\) current (I\(_{Ca,L}\) ). For the experiment shown in Figure 6e and 6g, the solution had Na\(^{+}\) and Ca\(^{2+}\) replaced with Li\(^{+}\) and Ni\(^{2+}\), and was K\(^{+}\)-free. Superfusing solutions were rapidly changed using a solenoid-controlled heated switching device. \(^2\) The pipette solution contained (in mmol/L): K glutamate 125; KCl 19; MgCl\(_2\) and CaCl\(_2\) 2; HEPES 10; MgATP 5; NaCl 10; HEPES 10; pH 7.25; and 50 \(\mu\)mol/L indo-1 (pentasodium salt, Calbochem). The liquid junction potential between the pipette and bath was corrected post hoc.

NCX Measurement With [Ca\(^{2+}\)]\(_{i}\) Buffer
In Figure 6a, the NCX current was measured selectively as described. \(^2\) The external solution was K\(^{+}\)-free (to block the inward rectifier K\(^{+}\) current and the Na\(^{+}/K\(^{+}\) pump) and also contained

[Image 100x300 to 460x516]
100 μmol/L niflumic acid, 10 μmol/L strophanthin (Na+/K+ pump inhibitor), and 10 μmol/L nitrendipine (dihydropyridine antagonist). The intracellular (pipette) solution contained (mmol/L): CsCl 110; NaCl 20; MgCl 2 0.4; MgATP 5; glucose 5; HEPES 5; CaCl 2 1.75; and BAPTA 5. The mixture of BAPTA and Ca gave a free [Ca] of 112 nmol/L (WinMaxC, Ver. 2.4; Chris Patton, Stanford University, Calif).

**XIP Synthesis**

XIP (RRLLFYKVVKYRAGKQRG) was synthesized by the Biosynthesis and Sequencing Facility, Department of Biological Chemistry, Johns Hopkins University, kept as 20 mmol/L stock in ethanol and added to the pipette solution (control experiments had equivalent amount of ethanol added, which had no effect on the parameters measured).

We did not observe any time-dependence of the effect of XIP, which reached steady-state as soon as we started recording (2 to 7 minutes from achieving the whole cell mode, at which time the indo-1 signal had reached a steady level). Statistics shown are mean ± SEM, with P<0.05 as the criterion for statistical significance.

**Results**

Because there is currently no selective, externally applicable inhibitor of NCX (available compounds10,11 or inorganic blocking cations12 are either nonselective14,15 or preferentially block reverse-mode exchange10), we compared cellular responses in the absence and presence of exchange inhibitor peptide14 (XIP), added directly to the intracellular solution. XIP has been shown to be an effective NCX inhibitor under various conditions.12,14,15

**Effect of XIP on Steady-State [Ca\textsuperscript{2+}] Transients**

Cardiac cells isolated from normal (N) or failing (F) hearts were subjected to trains of depolarizations to assess the main mechanisms of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR), ie, trigger Ca\textsuperscript{2+} entry through L-type Ca\textsuperscript{2+} channels (I\textsubscript{Ca,L}), the rate of rise (ΔCa/Δt) and amplitude (ΔCa) of the [Ca\textsuperscript{2+}], transient, and the SR Ca\textsuperscript{2+} load (C\textsubscript{SR}, measured as the integral of NCX current during caffeine application,2 see later; Figure 1). Myocytes from failing hearts cells showed the characteristic Ca\textsuperscript{2+} handling deficit, with decreased [Ca\textsuperscript{2+}], transients and C\textsubscript{SR}, and no change in I\textsubscript{Ca,L} (Figures 1 and 2). Internal equilibration with 10 μmol/L XIP induced a large increase in the steady-state C\textsubscript{SR} and [Ca\textsuperscript{2+}], transients, without any effect on I\textsubscript{Ca,L} (Figures 1 and 2). A small increase in C\textsubscript{SR} was also seen in normal myocytes. At a concentration of 30 μmol/L, an additional increase in ΔCa was observed in both groups. In failing cells, ΔCa was increased 3.86-fold by XIP compared with the untreated group (Figure 2c).

Importantly, and somewhat unexpectedly, the enhancement of excitation–contraction coupling occurred without a significant change in diastolic [Ca\textsuperscript{2+}], (see later).
Effect of XIP on the \([\text{Ca}^{2+}]_i\) Staircase

Immediately after a caffeine-induced \([\text{Ca}^{2+}]_i\) release (which unloads the SR completely) and thus gives a similar starting point in all groups; repetitive square wave depolarizations induced a gradual increase in the \([\text{Ca}^{2+}]_i\) transient (positive staircase or “treppe”) in N, as a consequence of SR \([\text{Ca}^{2+}]_i\) loading. XIP slightly accelerated the pulse-dependent \([\text{Ca}^{2+}]_i\) transient for the first 10 pulses (Figure 3), which, after 20 to 30 pulses, led to the increased steady-state values shown in Figures 1 and 2. The positive staircase was characteristically absent in untreated F, but was fully restored with the addition of 10 or 30 \(\mu\text{mol/L}\) XIP (Figure 3b). Again, the increase in the amplitude of the \([\text{Ca}^{2+}]_i\) transient was associated with a maintained or slightly decreased diastolic \([\text{Ca}^{2+}]_i\) (Figure 3c and 3d).

Effect of XIP on \([\text{Ca}^{2+}]_i\) Decay

Because NCX is a major \(\text{Ca}^{2+}\) removal mechanism, especially in myocytes from failing hearts, we anticipated that XIP might decrease the rate of diastolic \([\text{Ca}^{2+}]_i\) decay and adversely affect cell relaxation. However, the results indicated the
reported by Schouten et al. 16 and attributed to SERCA inhibition obtained with these concentrations.

On closer inspection, the time constant of decay of [Ca²⁺] was not directly on XIP, but was secondary to the increase in activation (because it was sensitive to thapsigargin 17). The decay of [Ca²⁺] was not caused by XIP in itself, we could reproduce the effect in F when the [Ca²⁺] was reduced in the presence of caffeine were used as baseline values in determining ΔCa.

Contrary: at steady-state, the time constant of decay of [Ca²⁺], on repolarization to the holding potential (τCa⁰; representing here the combined NCX and SERCA actions) was accelerated by XIP in both groups (Figure 4a). This indicated that NCX inhibition may be associated with an unexpected increase in the rate of SR Ca²⁺ uptake (which was also consistent with the large increase in Ca₉₀).

On closer inspection, the acceleration proved to be dependent not directly on XIP, but was secondary to the increase in [Ca²⁺], (Figure 4b). In normal cells, during the development of the Ca²⁺ staircase (as in Figure 3), the increase in peak [Ca²⁺], was reproducibly associated with an acceleration of τCa⁰ (Figure 4c, white circles). A similar effect was previously reported by Schouten et al. 16 and attributed to SERCA activation (because it was sensitive to thapsigargin 17). The same relationship was found in failing cells, when the staircase was recovered in the presence of XIP (eg. Figure 4b for typical traces; Figure 4c, black circles for average data). Finally, clearly demonstrating that the acceleration of Ca decay was not caused by XIP in itself, we could reproduce the effect in F when the [Ca²⁺] transients were enhanced by an increase in external Ca²⁺ concentration, in the absence of XIP (Figure 4c, black diamonds).

Therefore, we conclude that NCX inhibition, by decreasing Ca²⁺ extrusion, induced an increase in cytosolic Ca²⁺, which had the effect of stimulating SERCA. Both NCX inhibition and the indirect SERCA stimulation were responsible for the full magnitude of the inotropic effect. This occurred with the maintenance (and even improvement) of relaxation in F in the presence of XIP (see Discussion).

Quantification of NCX Inhibition

The large positive inotropic effect of 10 and 30 μmol/L XIP in F required an estimation of the actual degree of NCX inhibition obtained with these concentrations.

Because NCX is the major Ca²⁺ transporter during caffeine-induced Ca²⁺ release, the time constant of [Ca²⁺] decay (τCa⁰) in the presence of caffeine is a measure of NCX activity (Figure 1). However, we were unable to show a significant effect on τCa⁰ for caffeine-induced Ca²⁺ transients with 30 μmol/L XIP (Figure 5a). An alternative measure of NCX activity in these experiments is the amplitude of the inward NCX current, plotted as a function of [Ca²⁺]. This relation was also not significantly reduced by 30 μmol/L XIP in either group (Figure 5b; n values: N, 5/6; N +30XIP, 9/5; F, 4/3; F +30XIP, 3/3; failing group data not shown). Because XIP has been demonstrated to be an effective NCX inhibitor under various conditions, 12,14,15 we hypothesized that the specific conditions associated with the caffeine experiments may have masked the inhibitory effect (see Discussion). Therefore, we persisted in investigating this question by performing 2 additional experiments.

In the first, we measured NCX activity as the Ni²⁺-sensitive current elicited by depolarizations from −40mV to various potentials (as described 2). Consistent with previous results, 12 in these conditions, 30 μmol/L XIP inhibited NCX by ~67% (at +80mV, Figure 6a and 6b) in both N and F, and the block was mode-independent.

We also estimated the degree of NCX inhibition in the minimally Ca²⁺-buffered conditions that were used for the excitation–contraction coupling experiments shown in Figures 1 through 3. With SR Ca²⁺ uptake (and, thus, indirectly, Ca²⁺ release) blocked by thapsigargin, membrane depolarizations from −80 to +100 mV elicited reverse-mode NCX-mediated [Ca²⁺] increases (Figure 6c and 6d). Under these conditions, 10 and 30 μmol/L XIP induced 23% and 27% NCX inhibition, respectively (Figure 6c and 6d).

Selectivity of NCX Inhibition

XIP has been reported to inhibit both the sarcolemmal and SR Ca²⁺ pumps in vitro. 18 Therefore, it was important to establish that reversal of the failing phenotype was caused by a selective effect on NCX. In the same experimental conditions as for Figures 1 through 5, the time constants of [Ca²⁺], decay attributable to the SR Ca²⁺ pump and other, slower, mechanisms (including the plasmalemmal Ca²⁺ ATPase, PMCA, and mitochondrial Ca²⁺ uptake) were assessed using caffeine applications in Na⁺-free and Ca²⁺-free solution 19 (see Figure 6); 30 μmol/L XIP did not inhibit either transporter (Figure 6c and 6g). The previously reported sensitivity of the pumps to XIP was not observed here, likely because of differences in the experimental conditions. For example, Enyedi et al. 18 measured PMCA and SERCA in isolated membrane vesicles from rabbit erythrocyte and skeletal muscle preparations, respectively, and after proteolytic activation of PMCA.

Modulation by the Membrane Potential

The present experiments were designed to assess Ca²⁺-induced Ca²⁺ release at the maximum Ca²⁺ current (I⁰Ca,L) amplitude. However, an action potential-driven Ca transient would likely include a component of Ca²⁺ entry through the NCX, which is larger in F than in N. 20,21 Therefore, it was of interest to determine if the positive inotropic effect of XIP was also evident in F during trains of action potentials in current clamp conditions. Figure 7a and 7b show that action potential-triggered Ca²⁺ transients in F were significantly smaller than in N, and were significantly increased by 30 μmol/L XIP. In N, 30 μmol/L XIP also tended to increase...
the Ca$^{2+}$ transient amplitude, but the effect did not reach statistical significance.

**Modulation by Pacing Frequency**

Increased pacing frequencies are usually associated with an increase in diastolic [Ca$^{2+}$], because of the decrease in the diastolic interval; therefore, we tested whether NCX inhibition might exacerbate this increase. Figure 7c confirms that this was not the case. F groups treated with 30 μmol/L XIP (n=4/2) had similar diastolic [Ca$^{2+}$], levels, as compared with untreated N (n=5/2) when stimulated at frequencies between 0.5 and 3Hz. The increase in steady-state peak systolic [Ca$^{2+}$] was maximal between 1 and 2 Hz.

**Discussion**

**Effect of NCX Inhibition in Normal Canine Cells**

To maintain Ca$^{2+}$ balance at steady-state, cellular Ca$^{2+}$ extrusion via forward-mode NCX must match Ca$^{2+}$ entry via...
The absence of an increase in diastolic Ca\textsuperscript{2+} with XIP was, however, notable. One would have predicted that inhibiting Ca\textsuperscript{2+} extrusion would lead to slowed relaxation and an increase in diastolic steady-state [Ca\textsuperscript{2+}]. This was, a priori, an argument against the use of NCX inhibitors in heart failure. Serendipitously, we observed the opposite effect as a consequence of indirect [Ca\textsuperscript{2+}]-dependent stimulation of SR Ca\textsuperscript{2+} uptake.

Ca\textsuperscript{2+}-mediated SERCA activation was first described by Schouten in 1990\textsuperscript{6} and later coined “frequency-dependent acceleration of relaxation.”\textsuperscript{12,23} Although partial acceleration of SR Ca\textsuperscript{2+} uptake at high Ca\textsuperscript{2+} is expected because of the sigmoidal dependence of the transport kinetics on the concentration of the substrate,\textsuperscript{17} an additional activation has been ascribed to an allosteric regulatory mechanism. The nature of the [Ca\textsuperscript{2+}]-sensitive mediator is still unclear: a possible mechanism suggested by some,\textsuperscript{17} but not all,\textsuperscript{24} studies was calmodulin-dependent phosphorylation. Regardless of the mechanistic details, [Ca\textsuperscript{2+}]-mediated SERCA activation represents an effective autoregulatory mechanism that protects against cytosolic [Ca\textsuperscript{2+}], overload. It is also a positive feedback mechanism in which increased SR Ca\textsuperscript{2+} release and uptake potentiate each other, a likely explanation for the large inotropic effect induced by a relatively modest (23% to 27%) degree of NCX block in both N and F, frequency-dependent acceleration of relaxation has been shown to function in the failing hearts\textsuperscript{25} and may represent an attractive inotropic target by itself.

**Effect of XIP in Failing Heart Cells**

Because the balance between Ca\textsuperscript{2+} removal mechanisms is shifted toward more NCX-mediated Ca\textsuperscript{2+} extrusion in myocytes from failing hearts,\textsuperscript{8} one would expect that the same degree of NCX block would induce a more substantial increase in the [Ca\textsuperscript{2+}], transients of this group. This was exactly what we observed: a 27% inhibition of the NCX (which, in failing cells, is enhanced to ∼200% of normal)\textsuperscript{3} induced a 3.86-fold increase in the [Ca\textsuperscript{2+}], transient amplitude (Figure 2c).

Importantly, despite impaired basal SERCA function, the myocytes from failing hearts were able to autoregulate [Ca\textsuperscript{2+}], in response to NCX inhibition by taking-up and releasing more SR Ca\textsuperscript{2+}. Turning this argument around, one may speculate that the substantially lower (∼50% of normal) basal SR Ca\textsuperscript{2+} uptake rate in heart failure\textsuperscript{8} (Figure 6f), with only a 28% downregulation of pump protein,\textsuperscript{8} may be partially caused by the lower amplitude Ca\textsuperscript{2+} transients and the lack of Ca\textsuperscript{2+} stimulation. There may be a vicious cycle between decreased SR Ca\textsuperscript{2+} release and uptake in heart failure, which was interrupted by XIP. Analogously, the robust stimulation of SERCA activity by β-adrenergic stimulation in failing myocytes\textsuperscript{8} implies a low basal SERCA activity, but sufficient recruitable reserve capacity. The present findings motivate renewed efforts toward identifying the messenger involved in the Ca\textsuperscript{2+}-dependent stimulation of SERCA to clarify its therapeutic potential in heart failure.

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**Frequency-Dependent Acceleration of Relaxation**

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Experimental Assumptions and Limitations

The model used here has historically generated many insights into the physiopathology of heart failure. From the point of view of cellular Ca\(^{2+}\) handling, canine (as well as rabbit and guinea pig) myocytes resemble human cells, with 70% reliance on SERCA for Ca\(^{2+}\) decay, and 25% on NCX. This differs from small rodent species, such as rats and mice, in which NCX plays a lesser role. Hence, the effect of XIP may be substantially different in these animal models. The pacing-induced heart failure model resembles the human failing phenotype, with unchanged I\(_{Ca}\), increased NCX, and decreased SERCA. One difference, however, is that we have not observed an elevation of the diastolic levels of [Ca\(^{2+}\)], in F versus N, which may true in humans with end-stage heart failure. Therefore, one must be cautious about extrapolating the results to the human disease without further investigation.

The substantial increase in the [Ca\(^{2+}\)] transient in F suggested that XIP had a significant effect in these cells, but the caffeine-induced Ca\(^{2+}\) responses retained fast [Ca\(^{2+}\)] decay kinetics and large inward NCX currents (Figure 1c and Id). This apparent inconsistency begged the question of how much NCX was inhibited in these experiments. With alternative methods, we found that 30 \(\mu\)mol/L XIP inhibited NCX by 67% when Ca\(^{2+}\) was buffered to 112 nmol/L, and 27% when Ca\(^{2+}\) was allowed to oscillate freely between \(\approx 300\) and 500 nmol/L, suggesting that the apparent lack of effect of XIP in the presence of caffeine could be caused by the very high intracellular free Ca\(^{2+}\) concentrations reducing the effectiveness of the inhibitor. An alternative possibility is that the caffeine itself could be altering the extent of XIP block of the Na\(^{+}\)/Ca\(^{2+}\) exchanger, either directly or indirectly (eg, through its inhibition of phosphodiesterase activity). With respect to the notion that XIP inhibition could be diminished at high [Ca\(^{2+}\)], it may be noted that the allosteric Ca\(^{2+}\) binding sites and putative XIP interaction domains lie near each other on the large intracellular loop region of NCX, although competition between Ca\(^{2+}\) and XIP has not been observed in more isolated assay systems. Thus, further studies will be required to validate this hypothesis. If it proves true, however, this effect would constitute an additional safeguard against XIP-induced Ca\(^{2+}\) overload by excessive inhibition of Na\(^{+}\)/Ca\(^{2+}\) exchange.

Another important issue is that the overall contribution of NCX to Ca\(^{2+}\) handling, and to the effect of XIP documented here, is likely to be modulated by a number of cellular parameters in vivo. One is the specific contribution to the cellular Ca\(^{2+}\) transients of Ca\(^{2+}\) entry via reverse-mode NCX during the action potential. The overall effect of NCX inhibition would represent the balance between an increase in SR Ca\(^{2+}\) release and a decrease in NCX-mediated Ca\(^{2+}\) entry. Ca\(^{2+}\) entry through NCX is relatively more important in F, so this effect may account for the somewhat smaller (but still significant) effect of XIP on Ca\(^{2+}\) transients evoked by action potentials versus those evoked by voltage-clamp pulses. A second consideration is that intracellular Na\(^{+}\) levels have been reported to be increased in failing hearts, and this may have an impact on the effects of NCX inhibition in vivo, when Na\(^{+}\), is not controlled by the experimental conditions.

Therapeutic Relevance

The present findings suggest that NCX inhibitors may represent a new class of positive inotropic drugs in the treatment of congestive heart failure. Even in the absence of novel drug development, improvements in gene transfer technology in the near future may make myocyte-targeted XIP expression a feasible therapy. For the goals of this study, the NCX inhibitor was selective and mode-independent, but the positive inotropic effect could, theoretically, be facilitated by a preponderantly forward-mode NCX inhibitor and/or by block of PMCA (a lesser component of total Ca\(^{2+}\) efflux).

For clinical use, other possible effects of NCX inhibitors (on arrhythmogenesis, ischemia-reperfusion injury, etc) are in need of further investigation. While this is beyond the scope of the present article, it is worth noting that NCX inhibition will have both direct and indirect (ie, via the increase in Ca\(_{99}\)) effects, which sometimes may be contradictory. One example is the pro-arrhythmic mechanism involving delayed after depolarizations. Increased Ca\(_{99}\) (for example, in response to sympathetic overdrive) may predispose the heart to spontaneous SR Ca\(^{2+}\) release events, and trigger delayed after depolarizations as a result of forward-mode NCX current. In this case, NCX inhibition would be protective by decreasing the peak amplitude of the transient inward (I\(_{Ca}\)) current evoked by a spontaneous Ca\(^{2+}\) release. A similar point can be made about the effect of NCX inhibition on the action potential duration (another pro-arrhythmic mechanism). While XIP increased the duration of the action potential for stimuli evoking a similarly-sized [Ca\(^{2+}\)] transient, the XIP-induced enhancement of SR Ca\(^{2+}\) release shortens the action potential at steady-state, as a consequence of Ca\(^{2+}\) feedback on the action potential waveform. A protective effect against ischemia-reperfusion injury would also be expected in the presence of an NCX inhibitor.

In summary, the present results argue that partial inhibition of NCX is a powerful method for restoring excitation-contraction coupling in heart failure. This effect is brought about by an improvement of SR Ca\(^{2+}\) load and facilitation of the pulse-dependent positive Ca\(^{2+}\) staircase caused by a reduction in the amount of Ca\(^{2+}\) “stolen” from the cell on each beat by NCX. Secondary Ca\(^{2+}\)-dependent stimulation of the SR Ca\(^{2+}\) ATPase rate plays an additional important role in preventing diastolic [Ca\(^{2+}\)] overload. This study identifies NCX inhibition as a potential therapeutic strategy in congestive heart failure, a disease in which no effective long-term treatment is available, and offers a platform for the development of a new class of positive inotropic drugs. Finally, this study uncovers the importance of SR Ca\(^{2+}\) ATPase activation by [Ca\(^{2+}\)], whose recruitment acts synergistically with NCX block to increase both Ca\(^{2+}\) release and removal, normalizing both sides of the contractile deficit in heart failure.

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References


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Canine Pacing-Induced Tachycardia Heart Failure Model

Induction of heart failure was carried out as described previously\(^1\), using protocols approved by the Institution’s Animal Care and Use Committee. In brief, a VVI pacemaker (Medtronics) was implanted in mongrel dogs of either sex. Pacing at 240 bpm was maintained for 3-4 weeks, during which time the animals developed typical symptoms of heart failure including lethargy, loss of appetite, ascites, etc. Hemodynamic decompensation was confirmed by recording left ventricular (LV) pressure waveforms (under anesthesia with 25 mg/Kg tiopental) prior to sacrifice using a micromanometer - tipped LV catheter inserted through the right femoral artery. An increased end-diastolic pressure (>20mmHg), slowed rate of pressure rise and slowed relaxation rate were evident in F\(^2\).

Isolation of midmyocardial cardiomyocytes

After left lateral thoracotomy, the heart was perfused with ice-cold cardioplegic solution, containing (mmol/L): KCl 104; NaCl 32; NaHCO\(_3\) 10, taurine 10, BDM (butanedione monoxime) 20, pH 7.4, and quickly excised. The region of the left ventricular free wall perfused by the left anterior descending coronary artery was excised and perfused at 12 ml/min. The basic Ca\(^{2+}\)-free isolation solution contained\(^3\), in mmol/L : NaCl 130; KCl 4.5; MgCl\(_2\) 5; HEPES 23; glucose 21; taurine 20; creatine 5; NaH\(_2\)PO\(_4\) 1; Na pyruvate 5; pH 7.25 (titrated with NaOH), at 37\(^\circ\)C, oxygenated with 100% O\(_2\). The cardiac muscle was perfused in sequence\(^4\) with: 1) isolation solution with added 8 mmol/L EGTA for 15 min.; 2) isolation solution with 50 \(\mu\)mol/L Ca, 1 mg/ml collagenase (type I, 255 U/mg, Worthington Biochemical Corp., Freehold, N. J.) and 0.1 mg/ml protease (type XIV, Sigma Chemical Co., St. Louis, MO) for 12 min., and 3) isolation solution containing 100 \(\mu\)mol/L Ca and 10 mmol/L BDM for 6 min. for washout. Chunks of well-digested ventricular tissue from the midmyocardial layer of the ventricle were dissected out (after removing the epicardial and endocardial layers) and cells were mechanically disaggregated, filtered through nylon mesh and stored in modified Tyrode’s solution containing 1 mmol/L Ca. The procedure yielded Ca\(^{2+}\)-tolerant quiescent myocytes which survived well for up to 8 hours.
Single-cell electrophysiology studies

Cells were placed in a heated chamber on the stage of an inverted fluorescence microscope (IX70, Olympus, Inc.) and superfused with a physiological salt solution (see Methods). All experiments were carried out at 37 °C. We used a custom-built heated device capable of rapidly changing the external solution around the cell, while keeping the temperature at 37 °C. Borosilicate glass pipettes of 1-4 MΩ tip resistance were used for whole-cell recording with an Axopatch 1D amplifier coupled to a Digidata 1200A personal computer interface (Axon Instruments, Foster City, CA) using custom-written software.

\[ [Ca^{2+}]_i \text{ measurement} \]

\[ [Ca^{2+}]_i \text{ measurement was performed as described previously}^4 \text{ using the K}^+ \text{ salt form of indo-1. Cellular autofluorescence was recorded before rupturing the cell-attached patch and subtracted prior to determining R (ratio of 405nm emission/495nm emission). Ca}_i \text{ was calculated according to the equation Ca}_i = K_d \times \beta \times [(R \text{ – } R_{\text{min}})/(R_{\text{max}} \text{ – } R)]]^5, \text{ using a } K_d \text{ of 844 nmol/L}^6, \text{ and experimentally determined } R_{\text{min}} = 1, R_{\text{max}} = 4 \text{ and } \beta = 2. \]

The main experimental protocol

After establishing the whole cell mode, and capacitance compensation, cells were paced at a slow rate (0.1 Hz) until the indo-1 signals reached steady-state (usually 2-7 min), signifying sufficient diffusion of the pipette solution into the cell. The experimental protocol was then started and consisted of a train of 0.5 sec depolarizations from -80mV to +10mV, applied at 0.5 Hz until the [Ca^{2+}]_i transients reached steady-state. At this point 30 µmol/L tetrodotoxin (Na^+ channel blocker) was rapidly applied for a few beats, to allow a better estimation of the peak of the L-type Ca^{2+} current (I_{Ca,L}). The short TTX application did not affect the amplitude and rate of rise of the [Ca^{2+}]_i transients. TTX was then washed off, the train of depolarization stopped and caffeine was rapidly applied to measure sarcoplasmic reticulum Ca^{2+} load (Ca_{SR}).

During caffeine application, [Ca^{2+}]_i rose rapidly and then decayed exponentially, extruded (mainly) by NCX, which generated an inward current (Fig 1). In some experiments, 10 sec after caffeine wash-off, caffeine was reapplied. The second caffeine application produced no change in [Ca^{2+}]_i, reassuring that all SR Ca^{2+} was released with the first application. The total Ca^{2+} load of the SR was estimated from the integral of the NCX
current ($I_{NCX}$), which represented the charge moved by NCX while extruding Ca$^{2+}$. $I_{NCX}$ was further divided by the elementary charge ($1.6 \times 10^{-19}$ C) and Avogadro’s number ($6.023 \times 10^{23}$), giving the number of moles of Ca$^{2+}$ extruded. Cell volume was estimated from cell capacitance, divided by the membrane specific capacitance (1 μF/cm$^2$) and a typical cell surface/volume ratio of 0.5 μm$^{-1}$ (cf. ref.7). Cytosolic (non-mitochondrial) volume is 65% of cell volume. In the end, we used the equation $[Ca_{SR}] (\mu\text{mol/L}) = 76 \times I_{NCX} (\text{pC}) / \text{cell capacitance (pF)}$. The factor “76” combines all the constants used. Please note that the unit (μmol/L) refers to μmoles Ca$^{2+}$ stored in the SR divided by the total cell volume (in L).

Consistent with previous studies$^{1,2,4}$, cell capacitance and therefore the calculated cell volume were similar in F and N cells.

References

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