Muscarinic Modulation of the Sodium-Calcium Exchanger in Heart Failure

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Background—The Na-Ca exchanger (NCX) is a critical calcium efflux pathway in excitable cells, but little is known regarding its autonomic regulation.

Methods and Results—We investigated β-adrenergic receptor and muscarinic receptor regulation of the cardiac NCX in control and heart failure (HF) conditions in atrially paced pigs. NCX current in myocytes from control swine hearts was significantly increased by isoproterenol, and this response was reversed by concurrent muscarinic receptor stimulation with the addition of carbachol, demonstrating “accentuated antagonism.” Okadaic acid eliminated the inhibitory effect of carbachol on isoproterenol-stimulated NCX current, indicating that muscarinic receptor regulation operates via protein phosphatase–induced dephosphorylation. However, in myocytes from atrially paced tachycardia-induced HF pigs, the NCX current was significantly larger at baseline but less responsive to isoproterenol compared with controls, whereas carbachol failed to inhibit isoproterenol-stimulated NCX current, and 8-Br-cGMP did not restore muscarinic responsiveness. Protein phosphatase type 1 dialysis significantly reduced NCX current in failing but not control cells, consistent with NCX hyperphosphorylation in HF. Protein phosphatase type 1 levels associated with NCX were significantly depressed in HF pigs compared with control, and total phosphatase activity associated with NCX was significantly decreased.

Conclusions—We conclude that the NCX is autonomically modulated, but HF reduces the level and activity of associated phosphatases; defective dephosphorylation then “locks” the exchanger in a highly active state. (Circulation. 2007;115:1225-1233.)

Key Words: calcium ■ electrophysiology ■ heart failure ■ receptors, adrenergic, beta ■ sodium

The cardiac Na-Ca exchanger (NCX), a protein found in the sarcoplasmic membrane, acts as the major Ca\(^{2+}\) efflux path and is an important Ca\(^{2+}\) handling protein regulating intracellular Ca\(^{2+}\) in excitation-contraction coupling in the heart. The NCX plays an important role in pathological states as well, acting as a major Ca\(^{2+}\) entry site during ischemia/reperfusion.\(^1\) In heart failure (HF), the NCX is significantly upregulated in both human\(^2\) and animal models,\(^3,4\) which may significantly impair cardiac contractility by reducing sarcoplasmic reticular Ca\(^{2+}\) content via premature Ca\(^{2+}\) efflux.\(^3\) It may also promote unstable repolarization with early and/or delayed afterdepolarizations, triggering fatal ventricular arrhythmias.\(^6\)

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Cardiac output is controlled on a beat-to-beat basis by the interaction of the sympathetic and parasympathetic nervous systems. Cardiac sympathetic stimulation activates the β-adrenergic receptor (β-AR), whereas parasympathetic stimulation acts on cardiac muscarinic receptor (M-2) systems. These signaling systems interact to regulate cardiac contractility and rate by modulating critical effector proteins\(^7\) via “accentuated antagonism,”\(^8\) a phenomenon in which the effect of sympathetic stimulation is rapidly reversed by parasympathetic stimulation despite continued application of the initial stimulus. Accentuated antagonism allows rapid increases in cardiac output in response to physiological challenge while preventing toxicity from excess adrenergic tone.\(^9\) In HF both β-AR and M-2 cardiac responsiveness are depressed, contributing to lost cardiac reserve, increased arrhythmic susceptibility,\(^10\) and death. However, the underlying cellular mechanism of these phenomena is far from clear. Some studies have attributed this loss of autonomic responsiveness to the desensitization of β-AR receptors and/or downregulation of signal transduction in HF.\(^11,12\) However, recent work suggests that the L-type Ca\(^{2+}\) channel and ryanodine receptor are tonically phosphorylated (“hyperphosphorylated”) at baseline in failing human myocytes,\(^13-16\) which could contribute to depressed...
β-AR responsiveness in cardiac function in HF. Some investigators, but not all, have suggested that the NCX is regulated by β-AR17-19 and M-2 systems.20 Schulze et al21 reported that the NCX in heart is found in a macromolecular complex containing protein kinase A (PKA), protein kinase C, and the protein phosphatases PP1 and PP2a. We recently reported that the NCX current in HF was increased in the basal state,22 manifesting blunted β-AR regulation, and that these alterations are due to NCX hyperphosphorylation in HF.23

The effect of hyperphosphorylation on the muscarinic modulation of the affected proteins has not been investigated previously. In the present study, we investigated β-AR and M-2 regulation of the NCX in both control and HF states using a pacing-induced failing pig model. Our new model is similar to that reported previously except that the present study was performed in atrially (as opposed to ventricularly) paced animals. Rapid atrial pacing results in ventricular dilatation, neurohormonal activation, and loss of β-AR responsiveness similar to other HF models.24,25 The change in protocol was introduced for the following reasons: ventricular pacing bypasses the His-Purkinje conduction system and alters the normal pattern of ventricular depolarization, whereas atrial pacing does not (in the absence of conduction system disease).26 Altered ventricular depolarization has numerous effects on cellular protein expression, including the distribution of gap junctions27 and NCX protein density (David Rosenbaum, MD, PhD, personal communication, June 6, 2005). Ventricular pacing per se may introduce phenotypic changes in ventricular myocytes that confound our ability to assess the effect of HF. Additionally, ventricular pacing causes significant changes in ventricular repolarization, preventing a meaningful assessment of the impact of HF on the surface ECG and T wave. Using our refined HF model, we found that in HF the NCX was highly active and displayed markedly depressed β-AR and M-2 responsiveness. The common pathway of these alterations is downregulation of protein phosphatase expression and activity associated with the NCX, resulting in defective NCX dephosphorylation in HF.

Methods

Pacing-Induced Pig HF Model

Induction of HF and ventricular cardiomyocyte isolation and culture were performed as described previously,22,23 with the use of protocols approved by the university’s Institutional Animal Care and Use Committee, except that the animals were atrially rather than ventriculally paced. The investigation conforms 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). In brief, Yorkshire pigs (Animal Biotech Industries, Danboro, Pa) of either sex were anesthetized with thiopental sodium (10 mg/kg IV) to allow tracheal intubation (Animal Biotech Industries, Danboro, Pa) of either sex were anesthetized with thiopental sodium (10 mg/kg IV) to allow tracheal intubation.

Detection and Quantification of the Phosphatases

The total protein from ventricles of normal and failure pig hearts was extracted, and the Western blots were prepared as previously described.21,23 The NCX antigen complex NCX antibody/Protein A

Cardiomyocyte Isolation and Culture

On development of severe left ventricular dysfunction, animals were euthanized with pentobarbital sodium. The hearts were harvested by left lateral thoracotomy and immersed in ice-cold saline. The region of ventricle perfused by the left anterior descending coronary artery was excised, culminated, and perfused at 15 mL/min for 10 minutes with nominally Ca2+-free modified Tyrode’s solution (in mmol/L: NaCl 138, KCl 4, MgCl2 1, NaH2PO4 0.33, glucose 10, and HEPES 10 [pH 7.3 with NaOH] at 37°C and oxygenated with 100% O2). Perfusion was continued with the same solution but containing 0.24% (wt/vol) collagenase type I (Sigma, St Louis, Mo) and 0.028% protease XIV (Sigma) for 12 minutes and then for 10 minutes with washout solution with 0.1 mmol/L CaCl2 and 0.02% albumin. Sections of well-digested ventricular tissue from the midmyocardial layer of the ventricle were excised, and cells were mechanically dissociated and resuspended in buffers of gradually increasing [Ca2+]. To remove dead myocytes and residual contaminating cell types, the myocyte suspension was centrifuged through a discontinuous Percoll gradient, usually resulting in >90% rod-shaped cells. To test whether the myocytes to recover from enzymatic digestion, the cells were cultured overnight at 37°C in serum-free medium 199, supplemented with 5 mmol/L carnitine, 5 mmol/L taurine, 100 µg/mL penicillin, 100 units/mL streptomycin, and 0.25 µg/mL amphotericin. We have found that this approach increases the rate of successful giga-seal formation without significantly altering phenotype with regard to NCX function and β-AR regulation.

Electrophysiology

Whole-cell recordings were obtained at 37°C with the use of standard patch-clamp techniques. Membrane current was assessed by use of an Axopatch-100A amplifier and a 1/100 CV-3 head stage (Axon Instruments, Experimental control, data acquisition, and data analysis were accomplished with the use of the software package Pclamp 8.0 with the Digidata 1200 acquisition system (Axon Instruments). Patch pipettes were pulled from thin-walled glass capillary tubes and heat polished. The electrode resistance ranged from 1 to 2 MΩ. The external solution contained the following (in mmol/L): NaCl 145, MgCl2 1, HEPES 5, CaCl2 2, CsCl 10, Cs2SO4 10, taurine, 100, and glucose 10 (pH 7.4, adjusted with NaOH). Ouabain (0.02 mmol/L) and nifedipine (0.01 mmol/L) were added to the solution. The full scale of β-adrenergic stimulation was achieved by addition of isoproterenol (2 µmol/L). The internal solution contained the following (in mmol/L): CsCl 65, NaCl 20, Na2ATP 5, CaCl2 6, MgCl2 4, HEPES 10, tetracethyl ammonium chloride 20, EGTA 0.1, and ryanodine 0.05 (pH 7.2, adjusted with CsOH). In a separate set of experiments, protein phosphatase type 1 (PP1) (10 U/mL) was added to the internal solution to explore the role of tonic phosphorylation of the NCX in the increased basal I_{NCX} in HF. Membrane currents were elicited with the use of standard voltage ramp protocol. From a holding potential of −40 mV, a 100-ms step depolarization to +80 mV was followed by a descending voltage ramp (from +80 mV to −120 mV at 100 mV/s). The protocol was applied every 10 seconds. I_{NCX} was measured as the Ni-sensitive current. Ni2+ (5 mmol/L) was added to define the fraction of current that derives from NCX (the difference between total current and post-Ni2+ identity of the Bidirectional, Ni-Sensitive Current

To test whether our recordings were significantly contaminated by the cAMP-dependent Cl− current (CFTR) or the Ca-activated Cl− current, we applied niflumic acid (100 µmol/L, Ca-activated Cl− blocker) and glibenclamide (100 µmol/L, CFTR blocker). In separate experiments, we tested whether reducing extracellular Cl− (from 145 to 8 mmol/L) and whether removing extracellular Ca2+ would rapidly and reversibly suppress the isoproterenol-stimulated current.

Detection and Quantification of the Phosphatases

The total protein from ventricles of normal and failure pig hearts was extracted, and the Western blots were prepared as previously described.21,23 The NCX antigen complex NCX antibody/Protein A
Sephrose was obtained and prepared for Western blots and then immunoblotted with PP2α antibody (BD Biosciences, San Jose, Calif). After the PP2α in the immunoprecipitated NCX complex was identified, the same nitrocellulose membrane was reprobed with NCX antibody after the membrane was stripped of antibodies from the previous experiment. Similarly, another Western blot membrane was immunoblotted with PP1 antibody (Upstate Cell Signaling Solutions, Charlottesville, Va) and then with NCX antibody. With the use of ECL systems (GE Healthcare, Lake Placid, NY), the results were recorded on Biomax Kodak film. The images in the negative films were scanned and analyzed with the use of ImageQuant software (GE Healthcare Systems). Additionally, the β-1, β-2, and muscarinic receptor proteins were immunoprecipitated with the use of the appropriate antibody and quantified by Western blot.

Phosphatase Assay
To analyze the total activity of the phosphatases in heart extract and the phosphatases associated with the NCX complex, paranitrophenyl phosphate was used as a substrate, and the measurements were made following the protocol of the Phosphatase Assay Kit (Upstate Cell Signaling Solutions, Lake Placid, NY). Phosphatase activity for the normal and failure heart extracts (each containing 35 μg of total protein) was estimated. The phosphatase activity associated with NCX was determined for proteins binding to NCX. The NCX macromolecular complex was obtained by incubating 1.2 μL of antibody serum (r11-13) with the concentrated heart extract of 350 μL overnight. The NCX complex was washed thoroughly in 800 mL of the wash buffer.28 Enzyme activity was measured for 10 minutes at 37°C. The absorbance was measured at 405 nm. The phosphatase activity in normal heart was considered to be 100% and was compared with change in HF activity.

Protein Phosphatase Activity of PP1 and PP2a
The phosphatase activity of normal and failure heart extracts was determined with the use of the specific phosphopeptide (KRPtITRR) as a substrate for PP1 and PP2α phosphatase enzymes. The phosphate released was visualized with the use of malachite green following the manufacturer’s instructions (Serine/Threonine Phosphatase Assay Kit, Upstate Cell Signaling Solutions, Charlottesville, Va). The solutions were incubated for 20 minutes at room temperature, and color development was measured at 620 nm. The amount of enzyme activity was compared with those measured by the para-nitrophenyl phosphate method.

Statistical Analysis
Data are presented as mean±SEM. Continuous variables were compared by paired or unpaired t test. A probability value of <0.05 was regarded as a statistically significant finding.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Identity of the Bidirectional, Ni-Sensitive Current
During the voltage clamp protocol, the membrane potential of the cells was initially held at −40 mV to inactivate sodium channels. Cells were then depolarized to 80 mV to induce an outward current, as seen in Figure 1A, which reflects 3 Na⁺ flowing outward for 1 Ca²⁺ inward. The current becomes inward as the cell is hyperpolarized to −120 mV. The protocol is repeated in the presence of Ni²⁺, which blocks the NCX current, and this Ni-insensitive current is subtracted from the initial tracing to yield the Ni-sensitive bidirectional current. The protocol is then repeated in the presence of isoproterenol. If the induced current is indeed due to NCX activity, it should not be sensitive to blockers of Cl⁻ channels or to the concentration of Cl⁻ in the bath and pipette but should require the presence of Ca²⁺ in the bath.

Glibenclamide and niflumic acid, blockers of the CFTR and Ca-activated Cl⁻ currents, respectively, had no effect on the magnitude of the isoproterenol-stimulated, Ni-sensitive current whether given together (Figure 1B) or separately (not shown). In separate experiments, we found that reducing extracellular chloride (from 145 to 8 mmol/L) had no effect on the magnitude of the isoproterenol-stimulated current (Figure 1C), whereas removing Ca²⁺ rapidly and reversibly suppressed the isoproterenol-induced current (left). The mean peak outward current density in the presence or absence of [Ca²⁺]o is unchanged (right; P=0.98; n=9). D, Representative current tracing of hNCX after removal of Ca²⁺. Ca²⁺ removal rapidly and reversibly suppressed the isoproterenol-induced current (left). The mean peak outward current density in the presence or absence of [Ca²⁺]o is unchanged (right; P=0.05; n=7). These results are inconsistent with CFTR current and support the identity of the current as hNCX.
Blunted β-AR and M-2 Regulation of the NCX Current in HF

Recent evidence suggests that β-AR stimulation increases NCX activity in mammalian myocytes and that M-2 stimulation can modulate this effect. Figure 2A suggests that a representative current tracing of NCX from control myocytes in the basal state (blue), after exposure to isoproterenol (2 μmol/L, red), and after isoproterenol plus carbachol (5 μmol/L, green). Isoproterenol markedly increased outward and inward NCX, whereas carbachol reversed the increase in NCX induced by isoproterenol, consistent with “accentuated antagonism.” A2, The mean data of peak outward current at +70 mV (the number of independent experiments is shown in parentheses) as described in A1, confirming that isoproterenol (ISO) significantly increased NCX (*P<0.01, isoproterenol vs basal) and that carbachol (CCH) significantly reversed isoproterenol stimulation (#P<0.05, carbachol vs isoproterenol). B1, NCX from control myocytes. B2, The mean data of peak outward current (at +70 mV; the number of independent experiments is shown in parentheses) as described in A1, confirming that isoproterenol-induced current, demonstrating failure of muscarinic-accentuated antagonism. B2, The mean data of peak outward current (at +70 mV; the number of independent experiments is shown in parentheses) as described in A1, confirming that isoproterenol-induced current, demonstrating failure of muscarinic-accentuated antagonism. B1. NCX from control myocytes. This result suggests that the impaired β-AR and M-2 regulation is not due to reduced receptor number or uncoupling of the β-AR and M-2 signaling pathways. Rather, the defect must be downstream from the generation of cAMP and cGMP.

Muscarinic System Antagonizes β-AR Stimulation via Protein Phosphatase

Figure 4A shows that okadaic acid (1 μmol/L) significantly reversed the carbachol inhibition of isoproterenol-stimulated NCX current in control myocytes. This result suggests that a protein phosphatase is a crucial component for M-2 regulation of NCX in myocytes and argues for dephosphorylation as opposed to phosphorylation as a mechanism for M-2 modulation of the NCX. Furthermore, if a protein phosphatase is the mediator of M-2 modulation in the control state, it follows that infusion of exogenous protein phosphatase enzyme should reverse isoproterenol stimulation in HF in a manner similar to that of carbachol in the controls. In a separate experiment, dialysis of PP1 (10 U/mL) through the intracellular solution significantly inhibited NCX in basal and isoproterenol-stimulated conditions in failing myocytes but had no significant effect on basal current in control myocytes (Figure 4B). These data suggest that M-2 stimulation inhibits isoproterenol-stimulated carbachol did not result in a significant inhibitory effect on isoproterenol-stimulated NCX in HF cells (Figure 2B2). This is the first direct evidence of blunted M-2 regulation of the NCX current in HF. Our previous study in a ventricularly paced HF model demonstrated that increased basal NCX and reduced β-AR responsiveness are due to hyperphosphorylation of NCX in HF, but the mechanism of hyperphosphorylation and the interaction with the muscarinic system were not elucidated. Possible explanations for the failure of muscarinic modulation in HF include a decrease in M-2 receptor number, downregulated signal transmission, and/or altered protein phosphorylation state. These alternatives are investigated in the following experiments.

Altered Receptor Number and Downregulated Signal Transduction Are Excluded for Explaining Blunted β-AR and M-2 Regulation of NCX Current in HF

We found no evidence of a reduction in either muscarinic or β-adrenergic receptor protein expression in HF, suggesting that receptor number was not changed drastically in our model and pointing to a defect in signal transduction (see online-only Data Supplement). Stimulation of the cardiac muscarinic receptor (mainly M-2) is thought to activate soluble guanylyl cyclase, leading to an increase in intracellular cyclic GMP (cGMP). This increase in cytosolic cGMP could either activate cGMP-dependent protein kinase G (PKG) to phosphorylate effectors or activate a protein phosphatase to dephosphorylate PKA-induced phosphorylation. To identify the altered steps resulting in blunted β-AR and M-2 regulation of NCX in HF, we exposed failing cells to 8-Br-cAMP and 8-Br-cGMP to directly stimulate PKA and PKG. In control myocytes, cAMP (1 mmol/L) significantly increased NCX in a manner similar to that of isoproterenol, whereas cGMP significantly reversed this effect; cGMP also reversed the effect of isoproterenol (Figure 3). In failing myocytes, however, both cGMP effects were blunted, suggesting that the impaired β-AR and M-2 regulation is not due to reduced receptor number or uncoupling of the β-AR and M-2 signaling pathways. Rather, the defect must be downstream from the generation of cAMP and cGMP.

Figure 2. NCX responsiveness to isoproterenol and carbachol in control and failing myocytes. A1, A representative current tracing of NCX from a control myocyte in the basal state (blue), after exposure to isoproterenol (2 μmol/L, red), and after isoproterenol plus carbachol (5 μmol/L, green). Isoproterenol markedly increased outward and inward NCX, whereas carbachol reversed the increase in NCX induced by isoproterenol, consistent with “accentuated antagonism.” A2, The mean data of peak outward current at +70 mV (the number of independent experiments is shown in parentheses) as described in A1, confirming that isoproterenol (ISO) significantly increased NCX (*P<0.01, isoproterenol vs basal) and that carbachol (CCH) significantly reversed isoproterenol stimulation (#P<0.05, carbachol vs isoproterenol). B1, NCX from control myocytes. B2, The mean data of peak outward current (at +70 mV; the number of independent experiments is shown in parentheses) as described in A1, confirming that isoproterenol-induced current, demonstrating failure of muscarinic-accentuated antagonism. B1. NCX from control myocytes. This result suggests that the impaired β-AR and M-2 regulation is not due to reduced receptor number or uncoupling of the β-AR and M-2 signaling pathways. Rather, the defect must be downstream from the generation of cAMP and cGMP.
I NCX through activation of a protein phosphatase and that excessive protein phosphorylation in HF results in both increased basal activity and decreased \( \beta \)-AR responsiveness. A unifying hypothesis explaining these phenomena, as well as the failure of the NCX to respond to M-2 stimulation in HF, would be that the protein phosphatase associated with the NCX is significantly downregulated or inhibited in HF. To test this hypothesis, we measured protein phosphatase levels and activity in protein precipitated with the NCX complex.

### Protein Phosphatases Associated With NCX in HF

To test whether the protein phosphatases associated with the NCX are decreased in HF, we examined PP1- and PP2a-associated NCX with a specific antibody directed against PP1 and PP2a in NCX proteins immunoprecipitated by NCX antibody. The NCX proteins were immunoprecipitated by NCX antibody from control and failing heart tissues. The proteins in the NCX complex were separated with the use of PAGE and transferred to nitrocellulose membranes. These membranes were immunoblotted with PP2a antibodies, and the same blot was reprobed with NCX antibody after the membrane was stripped of antibodies from a previous experiment. Figure 5A is a representative Western blot of PP1 and PP2a in control and failing heart samples, showing that PP1 protein is significantly reduced in HF. Surprisingly, PP2a expression appears significantly increased in failing heart muscle compared with control in absolute terms (Figure 5B; \( P<0.05 \)). However, the total amount of NCX protein was increased in failing hearts by \( \approx 40\% \) (Figure 5B; \( P<0.05 \)). After normalization for the amount of NCX protein from control and failing hearts, group analysis confirmed that the mean amount of PP1 in failing hearts was 33% that in control hearts (\( P<0.01 \)), but PP2a levels were not altered with respect to NCX. To test whether this relative shift in protein expression would alter phosphatase activity, we assessed protein phosphatase activity associated with the NCX and in bulk myocardium with para-nitrophenyl phosphate and malachite green methods. Protein phosphatase activity associated with the NCX was significantly depressed in failing heart compared with control, but no significant difference existed between protein phosphatase activity in bulk myocardium between failure and control states (Figure 6). These results provide direct evidence that protein phosphatase activity associated with the NCX is downregulated in HF.

### Discussion

This is the first study to investigate the interaction of \( \beta \)-AR and M-2 regulation of the NCX in HF. The principal findings are as follows: (1) muscarinic receptor stimulation in control
myocytes significantly reverses β-AR stimulation of the NCX via activation of a protein phosphatase; (2) in HF, the NCX is “locked” in a relatively high activity state and is insensitive to both β-AR and M-2 regulation; (3) the common pathway of these alterations is downregulated protein phosphatase activity resulting in defective NCX dephosphorylation in HF; and (4) the profile of protein phosphatases associated with the NCX is significantly changed in HF with a reduction in PP1 but no change in PP2a. These results extend our previous findings that HF results in hyperphosphorylation of the NCX via activation of a protein phosphatase and represent an epiphenomenon of ventricular pacing.

Modulation of the NCX by the β-AR and Muscarinic Systems

Controversy exists regarding whether the cardiac NCX is modulated by PKA. The cAMP-dependent Cl− current (CFTR) and the Ca-activated Cl− current are reported to be Ni2+-sensitive and have reversal potentials similar to those of NCX. Lin et al have suggested that these conductances could contaminate our recordings of NCX, especially during stimulation of the β-AR, causing an overestimation of the effect of isoproterenol on the NCX. In the present study, we found no effect of significantly reducing extracellular chloride on the bidirectional, Ni2+-sensitive current in pig ventricular myocytes. Additionally, we found that the current was Ca2+-dependent, which is consistent with the NCX but not CFTR. Our result is consistent with other reports that although the CFTR conductance is highly represented in small animals, it is nearly or

Figure 5. Identification and quantification of protein phosphatases associated with NCX protein. The NCX macromolecular complex was immunoprecipitated from normal and failure heart extracts with the use of NCX antibody and Protein A Sepharose beads. The proteins in the NCX complex were separated with the use of PAGE and transferred to nitrocellulose membranes. These membranes were immunoblotted with PP2a antibodies, and the same blot was reprobed with NCX antibody after the membrane was stripped of antibodies from a previous experiment. A shows PP1 protein (top) in the complex immunoprecipitated by NCX antibody, PP2a (middle), and the NCX protein itself in the complex (bottom) in representative control (C) and HF (F) animals. B, The top panel shows the average amount of PP1 protein (±SEM) associated with the NCX complex from 6 HF and 5 normal animals. There was significant reduction of PP1 enzyme associated with NCX in HF (**P<0.01). There was a slight (but significant, *P<0.05) increase in the PP2a enzyme amount associated with NCX in the HF compared with normal hearts (middle). NCX protein (bottom) measured by Western blot was modestly but significantly increased in HF hearts compared with controls (P<0.05).
Further underscoring the significance of interspecies differences, Ginsburg and Bers found no evidence of isoproterenol stimulation of NCX in meticulously controlled experiments in non-failing rabbits. Although we have no experience with this model, one wonders whether differences in phosphatase activity may account for differences in response to isoproterenol; in such a case, an alternative approach using a phosphatase inhibitor might have yielded evidence of NCX augmentation. Our findings, furthermore, are consistent with those of other groups who found significant increases in NCX current after stimulation with isoproterenol. Further evidence that the NCX is regulated by the autonomic nervous system in a manner that is relevant to human disease is accumulating. Although stimulation of the M-2 receptor by carbachol in the absence of preceding β-AR stimulation has been shown to induce an indirect increase in the NCX current via subsarcolemmal Na⁺ gain, the present study confirms the observations of Zhang et al. that NCX activity is strongly depressed by muscarinic agonists administered during β-AR stimulation, but the mechanism of signal transduction was not previously elucidated. Investigating the L-type Ca channel, Jiang et al. reported that M-2 modulates β-AR stimulation via PKG-induced L-type channel phosphorylation, whereas Shen and Pappano have suggested that the primary response of cGMP is to activate protein phosphatases to dephosphorylate PKA-induced phosphorylation. To examine these putative mechanisms, we reasoned that if the muscarinic effect is modulated by phosphorylation of a separate site on the NCX in the presence of isoproterenol, then application of a nonspecific protein phosphatase inhibitor, ie, okadaic acid, should increase (or at least not inhibit) muscarinic modulation of the current by allowing further accumulation of phosphorylation. Alternatively, if M-2 stimulation results in activation of an NCX-associated protein phosphatase, inhibition of that phosphatase by okadaic acid should reverse the effect of M-2 stimulation and restore the current. We found that okadaic acid indeed reversed the carbachol effect, consistent with activation of a phosphatase as the mechanism of muscarinic modulation of the NCX. Finally, Katanosaka et al. have reported that the C-terminus of the Aβ of calcineurin, a phosphatase mechanistically implicated in cardiac hypertrophy, binds to the cytoplasmic loop of the cardiac NCX in hamsters. Prolonged phenylephrine exposure, which would stimulate the α-adrenergic system and act as a model of hypertension, resulted in inhibition of Na⁺,Ca²⁺-dependent Ca²⁺ efflux from isolated myocytes in a manner that was prevented by inhibition by calcineurin. This is consistent with differential phosphatase modulation by distinct pathological signaling mechanisms.

**Blunted β-AR and M-2 Regulation in HF**

Recent studies have suggested that elevated tonic phosphorylation (hyperphosphorylation) of calcium handling proteins such as the L-type Ca channel and the ryanodine receptor might contribute to increased basal Ca²⁺ permeability and reduced responsiveness to β-AR stimulation. In our previous study, we also found that the NCX is hyperphosphorylated in HF, suggesting that downregulated protein phosphatase might be the underlying mechanism. In the present study, we have demonstrated reduced PP1 protein and protein phosphatase activity associated with the NCX, providing a mechanism for NCX hyperphosphorylation in HF. The present study differs somewhat from our previous work in that these animals were atrially paced into HF. This change should avoid artifactual changes in myocyte phenotype due to abnormal depolarization;
however, the magnitude of the increase in the basal current due to HF appears to be 25% less than what was found previously despite similar increases in NCX expression. The most likely explanation is that rapid ventricular pacing induces a more severe HF phenotype because of the introduction of intraventricular and atrioventricular dyssynchrony that would not be appreciated by echocardiograms performed in sinus rhythm.

In the present study, we found that the M-2 receptor agonist carbachol loses its ability to inhibit β-AR stimulation on the NCX in HF. In contrast to the decreased numbers of β-AR receptors found in HF, the number of M-2 receptors is either unchanged or increased in HF. We found that application of cGMP fails to recover M-2 inhibition of β-AR signaling, confirming that the defect of muscarinic regulation is not at the M-2 receptor and/or G protein and must be downstream of cGMP. However, dialysis with PP1 significantly depressed both NCX current in basal conditions and after isoproterenol stimulation in HF. Furthermore, the protein phosphatase inhibitor okadaic acid eliminates carbachol after isoproterenol stimulation in HF. On the basis of these findings, we suggest that M-2 regulation of the NCX is dependent on protein phosphatase activity. On the basis of these findings, we suggest that both β-AR and M-2 dysfunction in HF are due to downregulation of protein phosphatase, principally PP1.

Downregulated Protein Phosphatase Associated With NCX in HF

The effect of HF on protein phosphatase expression and activity is clearly complex. Neumann et al. have reported that cardiac PP1 protein and mRNA levels were increased in HF, resulting in decreased phospholamban phosphorylation and depressed sarcoplasmic reticulum–ATPase function, whereas other groups have found decreased local protein phosphatase expression. Marx et al. found that the protein levels of both PP1 and PP2a associated with ryanodine receptor were reduced in HF. In the present study, we found that the NCX associates with protein phosphatases in a macromolecular complex in pig hearts, similar to findings in the rat. In contrast to the results of Marx et al. with respect to the ryanodine receptor, however, we found that PP1 expression is decreased, whereas PP2a associated with the NCX is unchanged in HF. Despite this shift in phosphatase profile associated with the complex, we found that total protein phosphatase activity associated with NCX is significantly depressed in HF compared with control (whereas total myocardial activity is unchanged). These results suggest that PP1 might play a principal role in NCX dephosphorylation in the control state, whereas PP2a is predominant in HF. Although it is generally believed that PP1 and PP2a have similar activity in terms of dephosphorylation of serine/threonine sites, the structure, activity, and regulation of these isoforms are distinct.

References

The sodium-calcium exchanger is the most important protein for removing calcium from the cytosol of cardiac muscle cells. Although calcium is needed to drive systolic contraction, excess calcium is associated with arrhythmias and cell death. Despite the importance of the sodium-calcium exchanger in maintaining cardiac homeostasis, the mechanisms controlling the activity of the exchanger are poorly understood. Sympathetic nervous system stimulation of the sodium-calcium exchanger by β-adrenergic agonists increases its activity in healthy pigs. In the present study in cardiac myocytes from healthy and heart failure pigs, the effect of agonists of the parasympathetic nervous system was tested and found to reverse the stimulatory effects of isoproterenol or cAMP analogues. In heart failure, however, the exchanger fails to respond to either sympathetic or parasympathetic nervous system activity but instead remains “locked” in a high activity state. This failure of modulation may contribute to increased myocyte calcium loss in heart failure, contributing to poor systolic contractile performance and arrhythmogenesis.
Muscarinic Modulation of the Sodium-Calcium Exchanger in Heart Failure
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_Circulation._ 2007;115:1225-1233; originally published online March 5, 2007; doi: 10.1161/CIRCULATIONAHA.106.650416

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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