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Molecular Regulatory Mechanisms of
the Sodium-Calcium Exchanger 1 in heart

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1. Abbreviations

AC  Adenyl cyclase
ADC  Analog-to-digital converter
ATP  Adenosine triphosphate
AVR  Aortic valve replacement
β-AR  β-adrenergic receptor
bpm  Beats per minute
$[\text{Ca}^{2+}]_i$  Cytosolic calcium concentration
CAD  Coronary artery disease
CABG  Coronary artery bypass
cAMP  Cyclic adenosine monophosphate
CaMKII  $\text{Ca}^{2+}/\text{calmodulin dependent kinase II}$
CBD1  Calcium binding domain 1
CBD2  Calcium binding domain 2
CFTR  Cystic fibrosis transmembrane conductance regulator
CICR  $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release
CLD  Catenin-like domain
CysPc  Cysteine protease core
DNA  Deoxyribonucleic acid
ECC  Excitation contraction coupling
FRET  Fluorescence resonance energy transfer
HEK  Human embryonic kidney
HF  Heart failure
HR  Heart rate
$I_{\text{ca}}$  L-type $\text{Ca}^{2+}$ channel
IkB  Inhibitor of kappa B
IP  Immunoprecipitation
ISO  Isoproterenol
KO  Knock-out
LTCC  L-type $\text{Ca}^{2+}$ channel
LV  Left ventricle
LVH  Left ventricle hypertrophy
Lys  Lysine
Met  Methionine
$[\text{Na}^+]_i$  Cytosolic sodium concentration
NCX  Sodium calcium exchanger
NF-κB  Nuclear factor kappa-light-chain-enhancer of activated B cells
NKA  Sodium potassium ATPase, Sodium potassium pump, Na$^+$/K$^+$ ATPase
NMR  Nuclear magnetic resonance
PC1  Protease core 1
PC2  Protease core 2
PEF(S)  Penta EF-hand domain (small subunit)
PEF(L)  Penta EF-hand domain (large subunit)
PKA  Protein kinase A
PKC  Protein kinase C
PLA  Proximity ligation assay
PLB  Phospholamban
PLM  Phospholemman
PMCA  Plasma membrane Ca$^2+$ ATPase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PP1</td>
<td>Protein phosphatase 1</td>
</tr>
<tr>
<td>PTMs</td>
<td>Post translational modifications</td>
</tr>
<tr>
<td>RYR2</td>
<td>Ryanodine receptor 2</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small-angle X-ray scattering</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>Sarcoendoplasmic reticulum Ca(^{2+}) ATPase, cardiac isoform</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>TMS</td>
<td>Transmembrane segment</td>
</tr>
<tr>
<td>TnC</td>
<td>Troponin C</td>
</tr>
<tr>
<td>XIP</td>
<td>Exchanger inhibitory peptide</td>
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2. List of publications

I. Full-length cardiac Na\(^+\)/Ca\(^{2+}\) exchanger 1 protein is not phosphorylated by protein kinase A

II. Molecular Basis of Calpain Cleavage and Inactivation of the Sodium-Calcium Exchanger 1 in Heart Failure

III. Development of a High Affinity Phospholemman Binding Peptide that Reverses Phosphorylated Serine 68 Phospholemman Inhibition of the Cardiac Sodium-Calcium Exchanger 1
3. Introduction

3.1. Heart failure

Heart failure (HF) is a complex and progressive disease that occurs when the heart is unable to supply adequate blood flow to the tissue at a normal filling pressure (1,2). HF can result in exercise intolerance and dyspnea (breathlessness). The severity of these features depends on stages of the HF. Its prevalence remains high despite remarkable improvement in HF therapy in last decades. Two common causes of HF are coronary artery disease and myocardial infarction. In addition, hypertension, aortic valve disease, cardiomyopathy, and idiopathic myopathy (cardiac failure in elderly) are risk factors participating in development of HF. Mortality in HF patients are usually due to contractile failure or ventricular arrhythmia.

HF can be categorized into systolic or diastolic HF (3). Systolic HF is caused by an impaired ventricular contraction (the heart muscle is unable to contract normally), while diastolic HF is due to a stiff ventricle caused by reduced ventricular compliance (e.g., as occurs with fibrosis) leading to impaired relaxation or impaired filling. Diastolic dysfunction is a major cause of HF. However, it is generally common that HF patients have a combination of both systolic and diastolic dysfunction. These patients tend to have worse prognosis than patients with either isolated systolic or diastolic dysfunction (4).

Pathologic cardiac hypertrophy (often left ventricular) occurs in response to pressure overload (aortic stenosis, hypertension), volume overload or after myocardial infarction. Pathological hypertrophy induced by pressure overload is often characterized as concentric hypertrophy, while eccentric hypertrophy is generally caused by volume overload (Figure 1).
Ventricular remodeling can be categorized into two major types that are physiological hypertrophy and pathophysiological hypertrophy. Physiologic hypertrophy is induced by exercise and pregnancy. Pathophysiologic hypertrophy can be either concentric or eccentric hypertrophy. Both concentric and eccentric hypertrophy can lead to HF. Figure is modified from Kehat 2010 (5).

Various cellular and molecular mechanisms are responsible for pathological myocardial remodeling and involve changes in cardiac myocytes, altered excitation-contraction coupling, aberrant expression of hypertrophic genes, changes in post-translational modifications of proteins, changes in extracellular matrix, necrosis and apoptosis of myocytes. Identification of the cellular and molecular changes is important for development of effective pharmacological strategies to treat HF patients (5-7). Several therapeutic interventions for treatment of HF have been reported. For example; 1) targeting hypertrophic signaling pathway in order to reverse pathophysiological ventricular remodeling, and 2) targeting ion handling proteins in order to improve Ca\(^{2+}\) cycling and thus cardiac contractility (8). However, development of potential drugs to treat HF remains challenging because the underlying mechanisms responsible for the transition from a pathophysiological hypertrophic heart to a failing heart are still enigmatic. In this thesis, we focus on identification of the molecular regulatory mechanisms of the cardiac sodium calcium exchanger 1 (NCX1).
3.2. Excitation-contraction coupling (ECC)

Excitation-contraction coupling is a process whereby an action potential activates cardiac contraction (Figure 2). During membrane depolarization, Na$^+$ ions enter the cell via voltage-gated sodium channels, leading to Ca$^{2+}$ influx via voltage-gated L-type Ca$^{2+}$ channels (LTCCs). Ca$^{2+}$ ions entering cardiomyocytes via LTCCs, bind to ryanodine receptor 2 (RYR2). The binding of Ca$^{2+}$ to RYR2 triggers Ca$^{2+}$ release from the sarcoplasmic reticulum (SR). This process called “Ca$^{2+}$ induced Ca$^{2+}$ release” or CICR. The released Ca$^{2+}$ ions bind to troponin C (TnC) on actin filaments initiating the interaction of actin filaments with myosin to generate contraction of the heart. For relaxation of the heart, Ca$^{2+}$ ions need to dissociate from the contractile apparatus and be transported into the SR by Ca$^{2+}$ ATPase-2a (SERCA2a) in SR. Remaining Ca$^{2+}$ ions in the cytosol will be extruded out from the cardiomyocytes through plasma membrane Ca$^{2+}$ ATPase (PMCA), the mitochondria Ca$^{2+}$ uniporter and NCX1 (forward mode).

![Figure 2](image-url)  
**Figure 2** Excitation–contraction (EC) coupling in normal hearts. Direction of Ca$^{2+}$ – and Na$^+$ ions are indicated by green and orange arrows, respectively. Figure is modified from © Louch
3.3. Sodium calcium exchanger 1

Sodium calcium exchanger (NCX) is a transmembrane protein that is responsible for ion transportation in most cells. The main function of NCX is to extrude one Ca\(^{2+}\) out from the cell in exchange for three Na\(^{+}\) (forward mode) (9). NCX can also operate Ca\(^{2+}\) influx (reverse mode) and the determinants of the directionality of net Ca\(^{2+}\) flux are the ion gradients across the membrane and membrane potential.

NCX has three isoforms; NCX1, NCX2 and NCX3. NCX1 is expressed abundantly in the heart, and it can also be found in other tissues including brain, kidney, lung and spleen depending on its alternative splice variants. NCX1.1 is the only splice variant that is expressed in the heart (10). NCX1 is found in sarcolemma and along T-tubules where it plays a major role in Ca\(^{2+}\) removal from the cell. NCX2 and NCX3 are expressed in brain and skeletal muscle tissues (11-13). There is about 70% identical in amino acid sequence between NCX isoforms and their molecular topologies are very similar (14).

3.3.1 Topology of NCX1

NCX1 consists of 970 amino acids with multiple hydrophobic segments (15,16). The first 32 amino acids at N-terminus (a signal peptide) are cleaved off during biosynthesis to produce the mature protein (17,18). The full-length mature protein of NCX1 contains 938 amino acids with a molecular mass of 120 kDa. In addition to the 120 kDa band, the purified NCX1 visualized by SDS-PAGE migrates also at approximately 140 and 70 kDa and the apparent size differences are due to intramolecular cross-linking of NCX1 proteins and proteolysis, respectively (19-21). Disulfide cross-linking studies suggest that NCX1 also can undergo dimerization and recent study using FRET technology revealed that NCX1 exists as a dimer in the membrane (21-23). However, the role of oligomerization of NCX1 remains to be determined (24). Current topology model based on cysteine susceptibility analysis and epitope tagging suggests that the mature NCX1 protein is consisting of 9 transmembrane segments (TMSs) (25,26) with a large hydrophilic intracellular loop (~550 amino acids) between TMS5 and TMS6 (Figure 3). However, recent crystal structure study revealed that an NCX from Methanococcus jannaschii (NCX_Mj) contains 10 TMSs (27).
NCX1 contains two conserved α-repeat regions (α-1 and α-2 repeat) which are critical for its transport function (28-30). The α-1 and α-2 repeats reside within the sequence spanning TMS2-3 and TMS7-8, respectively (Figure 3). The α-repeat regions are thought to form the ion binding pocket and to catalyze ion transportation across the plasma membrane (31).

Figure 3 Topology model of NCX1. TMSs, brown; XIP, pink; CLD, dark blue; CBD1, green; and CBD2, grey.

The cytoplasmic loop of NCX1 comprises of several specific regions such as XIP region, catenin-like domain (CLD), Ca\(^{2+}\) binding domain 1 (CBD1) and Ca\(^{2+}\) binding domain 2 (CBD2)(32). The XIP (eXchanger Inhibitory Peptide) region is at the N-terminus in the cytoplasmic loop, adjacent to TMS5, and is similar to a calmodulin binding site (16). The XIP region has been shown to have a regulatory role because a 20-amino-acid synthetic peptide corresponding to this sequence inhibits NCX1 function (33,34). However, the exact role of the XIP region remains elusive.

The catenin-like domain (CLD) is designated as the domain connecting the two Ca\(^{2+}\) binding domains (CBD1 and CBD2) with TMS5 and TMS6 (32,35). The CLD has been proposed to involve in signal transduction. Upon binding of regulatory Ca\(^{2+}\) to CBD1 and CBD2, CBDs undergo an rearrangement that induces an optimal conformation for transducing signals from CBDs to TMS domain via CLD thus activates NCX1 (32). Structural studies of CBD1 and CBD2 by Nuclear magnetic resonance (NMR) analyses reveal that upon Ca\(^{2+}\) binding, CBD1 acts as a primary Ca\(^{2+}\) sensor and it undergoes large conformational changes, while CBD2 seems to play an essential role to sustain NCX1 activity. The binding of Ca\(^{2+}\) to CBD2 is required to remove Na\(^{+}\)-dependent inactivation (36-38).
3.3.2 NCX1 knock-out (KO) and NCX1 overexpressing mice

Cardiac specific NCX1-KO (conditional) survived to adulthood with 20-30% depressed cardiac function despite 90% reduction in NCX1 expression (39-41). The study showed no compensatory changes in protein expression of other Ca\(^{2+}\) regulatory proteins. However, they found that LTCC current was 50% decreased with an unchanged SR Ca\(^{2+}\) release and a shorter action potential (AP). These observations suggested that a reduced Ca\(^{2+}\) clearance from the dyadic cleft (vicinity of LTCC and RYR2) in NCX1-KO mice increased local Ca\(^{2+}\) accumulation which then led to inactivation of LTCC. The facts that these NCX1-KO mice had a normal cardiac function with unchanged SR Ca\(^{2+}\) release and shorter AP, suggest a role of Ca\(^{2+}\) influx via NCX1 in ECC. NCX1 has been suggested to be involved in CICR by bringing Ca\(^{2+}\) into the cell by its reverse mode and possibly contributing to enhancement of ECC efficiency in mice (40,42,43). However, other reports that Ca\(^{2+}\) influx via NCX1 is not efficient enough to trigger CICR (44-47).

Contractility and ECC has also been studied in homozygous mice overexpressing NCX1 (48). These transgenic mice showed opposite changes to those reported in the NCX1-KO mice. These animals had a mild cardiac hypertrophy and they were prone to HF when treated with hemodynamic stress. The alterations including decreased Ca\(^{2+}\) transient, increased Ca\(^{2+}\) influx via LTCC and prolonged AP duration in myocytes overexpressing NCX1 indicate compensatory mechanisms in order to maintain normal contractility (43). Interestingly, the reduced ECC gain observed in the NCX1 overexpressing mice, resembles those observed in animal models of pressure-induced cardiac hypertrophy (49,50), supporting that NCX1 plays a role in adaptive/maladaptive mechanisms during abnormal changes in ECC and in the transition from hypertrophy to HF (48).

3.3.3 NCX1 in heart failure

Ca\(^{2+}\) removal by NCX1 is important to maintain normal Ca\(^{2+}\) homeostasis and cardiac contractility. Alterations in NCX1 function are implicated in defective ECC contributing to contractile dysfunction and arrhythmias which are major characteristics of HF (51). Several studies using aortic banding animal model (pressure overload induced hypertrophy) reported both no change and increase in level of NCX1 protein expression together with an increased NCX1 function (52) However, a decreased NCX1 function was also observed in some studies using the pressure overload induced hypertrophy model (53,54).
Increased level of both the NCX1 expression and activity has been reported in human failing heart tissue and in patients with end-stage HF. These changes are suggested to be the compensatory mechanisms for Ca\(^{2+}\) clearance and improvement of diastolic function (55-58). However, such compensatory role of NCX1 can be a two-edged sword because the increased NCX1 activity can contribute to both SR Ca\(^{2+}\) depletion and Ca\(^{2+}\) overload, which are two common features for contractile dysfunction in failing heart (52,59) (See more details in section 7.3). Ca\(^{2+}\) overload increases subsequent Ca\(^{2+}\)-dependent proteases activity such as calpains (60), leading to cleavage and dysregulation of several cardiac proteins (61).

However, evidences obtained from studies of NCX1 function using different animal models (pressure overload, volume overload and post-myocardial infarction leading to hypertrophy and HF) suggest that regulation of NCX1 activity appears to be influenced by other factors rather than a direct relationship with changes in its protein expression (62). Such factors are thought to be related to changes in NCX1 regulator proteins and/or changes in \([\text{Ca}^{2+}]_i\) and \([\text{Na}^+]_i\), due to altered ion transport systems, probably through SERCA2a, RYR2 and sodium-potassium pump; Na\(^+\)/K\(^+\) ATPase (NKA). Such changes in ion transport systems can occur concomitantly with or without changes in NCX1 expression during hypertrophy and HF (52). Identification of the mechanisms accompanying with changes in NCX1 expression and further modulating NCX1 activity provides crucial information about mechanisms underlying for contractile dysfunction and arrhythmogenesis.

NCX1 activity is tightly controlled by membrane potential, \([\text{Ca}^{2+}]_i\) and \([\text{Na}^+]_i\), thus changes in ion concentrations or ion fluxes mediated by other ion channels can potentially influence NCX1 properties. The NKA is an important mechanism transporting Na\(^+\) and K\(^+\) across cardiac sarcolemma and its primary function is to remove Na\(^+\) ions out of the cell (63). Loss of NKA activity increases \([\text{Na}^+]_i\) and high \([\text{Na}^+]_i\) favors Ca\(^{2+}\) influx mediated by NCX1 (in reverse mode) leading to Ca\(^{2+}\) overload (64). NKA consists of two subunits, a catalytic and small subunits (α- and β- subunits, respectively). The α-subunit contains Na\(^+\), K\(^+\) and ATP binding sites (65-68) and has two isoforms; α1 and α2. The NKA α2-isoform (NKA-α2) influences NCX1 activity (69-74), and both proteins co-localize in the plasma membrane along the t-tubules (66,72,75,76). Blockade of the α2-isoform by ouabain (inhibitor of NKA activity) reduces the rate of Ca\(^{2+}\) efflux through NCX1 (73) leading to slow relaxation (71,73). Down-regulation of NKA-α2 in HF disrupts the coupling functions between NKA and NCX1 (71). The role of NKA-α2 in regulating NCX1 activity has also been studied and confirmed using cardiac-specific transgenic mice overexpressing NKA-α2 (77). Taken together, these
observations suggest that the interplay between these two mechanisms is lost during HF and mechanisms regulating this functional link between NKA and NCX remain to be elucidated (71,78).

3.4. Molecular determinants of NCX1

3.4.1 Protein kinase A

Cardiac contraction is mediated by the sympathetic nervous system transmitting signals to cardiomyocytes via beta-adrenergic receptors (β-ARs) (79). Upon β-AR stimulation, catecholamines bind to β-ARs and thereby activate the adenylyl cyclase (AC) which in turn elevates the level of second messenger cAMP (cyclic adenosine monophosphate). cAMP will further activate the cAMP-dependent protein kinase A (PKA). PKA is an inactive tetrameric complex composed of a dimer of regulatory subunits (R) and two catalytic subunits (C) (Figure 4). The catalytic subunits are activated upon binding of two cAMP molecules to each R subunit causing the release of C subunits. The released C subunits will then phosphorylate its substrate proteins on serine and threonine residues. cAMP is degraded by phosphodiesterases.

![Figure 4](image)

**Figure 4** Schematic illustration of cAMP activation of PKA. Binding of cAMP to the regulatory subunit of PKA holoenzyme, causes the release of catalytic subunit. The catalytic subunit then becomes active, possessing protein kinase activity, and phosphorylates its substrates.

PKA regulates cardiac contraction and relaxation through phosphorylation of several proteins involved in the ECC (Figure 5). Phosphorylation of LTCC leads to increased Ca\(^{2+}\) current, whereas phosphorylation of the endogenous SERCA2a inhibitor; phospholamban (PLB), leads to increased SR Ca\(^{2+}\) uptake (80-82). In addition, PKA phosphorylates also troponin-I, causing reduction of Ca\(^{2+}\) sensitivity of myofilament (83). Hyper-phosphorylation of RYR2 by PKA is suggested to be responsible for SR Ca\(^{2+}\) leak, causing SR Ca\(^{2+}\) depletion.
in HF (84,85) and the SR Ca$^{2+}$ leak via PKA-hyper phosphorylated RyR2 has also been suggested to increase the risk of arrhythmias induced by NCX1 in rabbit HF myocytes (86-88). Noteworthy, PKA phosphorylation of NCX1 has been controversial for more than a decade (89,90). Although some studies report a direct PKA phosphorylation of NCX1, other studies suggest that the effect of β-AR signaling pathway on NCX1 is a secondary consequence (91-93).

Figure 5 Schematic illustration of β-AR activation and PKA phosphorylation of proteins involved in the ECC in the cardiomyocyte. Figure is modified from © Louch.

3.4.2 Phospholemman

Phospholemman (PLM) is a transmembrane protein containing 72 amino acid residues with an N-terminal cleavable signal peptide (Figure 6) (94). PLM belongs to the FXYD family of proteins and consists of an extracellular domain, a transmembrane (TM) domain and a COOH terminus residing in the cytosol. The FXYD protein family is defined by the amino acid sequence consisting of Phe-X-Tyr-Asp (FXYD) motif in the extracellular domain (95,96). The seven members of the FXYD family proteins in mammals are FXYD1 (phospholemman, PLM), FXYD2 (γ-subunit of NKA), FXYD3 (mammary tumor marker Mat-8), FXYD4 (channel inducing factor; CHIF), FXYD5 (related to ion channel RIC or dysadherin), FXYD6 (phosphohippolin) (97) and FXYD7.
PLM (FXYD1) is a major substrate of PKA and protein kinase C (PKC) in the heart and its physiological role depends on its phosphorylation status. PKA phosphorylates PLM at serine 68 and PKC phosphorylates serine 63, serine 68 and threonine 69 (98,99) (Figure 6). Phosphorylation at serine 68 (pSer68-PLM) contributes to 1) loss of its inhibitory effect on NKA (100-103) and 2) activation of its inhibitory effect on NCX1 (104,105). Phosphorylation of serine 68 in PLM has recently been shown to enhance palmitoylation at cysteine 40 and cysteine 42 leading to inhibition of NKA (106,107). Cysteine 42 can also be glutathionylated (108). In addition to its regulatory role on NKA and NCX1 activity, PLM is also reported to modulate activity of LTCC (109). However, the regulatory mechanisms need to be elucidated.

![Figure 6](image)

**Figure 6** Domain organization of the PLM (FXYD1) protein. Numbering of the FXYD1 sequence begins after the cleavable signal peptide domain (20 amino acids). The FXYD motif in the extracellular domain is underlined. The transmembrane domain is in grey. The palmitoylation sites at cysteine 40 and 42 and phosphorylation sites at serine 63, serine 68 and threonine 69 in the intracellular domain are in bold. Figure is modified from Feschenko 2003 (110).

Although there are several strong evidences suggesting that PLM is an endogenous NCX1 inhibitor (111), lack of a direct interaction between PLM and NCX1 and absence of direct effect of PLM on NCX1 activity have also been reported (112-114). Interestingly, PLM-deficient mice overexpressing PLM (S68E) (mimicking phosphorylation at serine 68) were reported to inhibit NCX1 without any effects on NKA activity, suggesting that PLM might be a direct regulator of NCX1 activity (115). A recent study of mice overexpressing PLM (S68E) showed that only slightly reduction in NCX1 activity (~34% inhibition) in these mice contributed to arrhythmias, cardiac hypertrophy, HF and 50 % mortality at 4-6 weeks of age, suggesting that constitutive overexpression of PLM (S68E) to be detrimental (116).
3.4.3 Calpain

Calpains are Ca\(^{2+}\) activated cysteine proteases and they belong to the papain superfamily with distinct active site cleft (117,118). Calpains are involved in a variety of biological processes such as proliferation, cell migration, differentiation, apoptosis and cell signaling (119,120). The calpain system is different from other proteolytic systems (such as proteasomes and lysosomal proteases) because of two reasons; 1) calpains modulate substrate activity and 2) calpains directly recognize their substrates (121).

The calpains are classified into conventional calpains and unconventional calpains. The conventional calpains are two ubiquitous expressed forms of calpain; \(\mu\)-calpain (calpain-1) and \(m\)-calpain (calpain-2). The difference between calpain-1 and calpain-2 is the requirement of calcium concentration (\(\mu\)M and mM, respectively) for their half-maximal activity \emph{in vitro} (122). The unconventional calpains are tissue-specific expressed, such as calpain-3, which is highly expressed in skeleton muscle, calpain-6 which is found in placenta and embryonic muscles, and calpain-8 which is a stomach-specific protease (123). The conventional calpains are heterodimers consisting of a catalytic subunit (large subunit, 80K) and a regulatory subunit (small subunit, 30K) (Figure 7). The regulatory subunit and calpastatin (specific calpain inhibitor) are important endogenous regulatory proteins of calpain. Deficiency in the regulatory subunit (30K) leads to embryonic lethality and down-regulation of both calpain-1 and calpain-2 catalytic subunit, suggesting that the regulatory subunit is involved in stabilization of catalytic subunit. The regulatory subunit is constituted of two regions; 1) the N-terminal Gly-rich domain and 2) the penta EF-hand domain (PEF (S)) (Figure 7). The catalytic subunit consists of four regions; 1) the N-terminal anchor helix, 2) the protease core domains (PC1 and PC2) constituting the CysPe domain, 3) the C2-like domain and 4) the penta EF-hand domain (PEF (L)).
Studies of the crystal structure of calpain suggest that upon activation, Ca$^{2+}$ binding to the CysPC domain (domain II) results in formation of an active site cleft. Unlike those observed in other papain-like cysteine proteases. The active site cleft of calpains has the width and depth that is preferentially for substrates with unstructured inter-domain exposed region (118). Several calpain substrates have been identified such as calcineurin, calmodulin-protein kinase, IκB, PKC, NCX1 and NCX3 (117,125-128). However, prediction of calpain cleavage sites and a clear defined rule for calpain substrate preferences remains elusive.

Calpain is involved Ca$^{2+}$ homeostasis in brain, cardiac and skeleton muscle and its activity is increased in various pathophysiological processes. Overexpression of its endogenous inhibitor, calpastatin, prevents brain ischemia by inhibiting calpain cleavage of NCX3, and thus reducing Ca$^{2+}$ overload in neurons (127). Calpain is also implicated in Ca$^{2+}$ dysregulation by modulating NCX1 activity in mitochondria (129), caveolae vesicles (130) and in Xenopus oocytes expressing exogenous NCX1 (131). Proteolysis of junctophilin (a protein anchoring T-tubules to SR) by calpain leads to the disruption of the triad junction, while calpain cleavage of α-fodrin (cytoskeleton protein) leads to collapse of membrane integrity, which further alters function of ion channels. Calpain cleavage of junctophilin and α-fodrin have been shown to be involved in the disruption of E-C coupling in skeletal muscle and heart (132,133).
4. Main aims

NCX1 is important for maintenance of normal Ca\(^{2+}\) homeostasis and contractility. Little is known about the molecular mechanisms regulating its activity. Identification of molecular mechanisms associated with the NCX1 protein complex might contribute to the development of more effective and specific therapeutic strategies for HF treatment. This thesis focuses on the regulatory role of PKA, PLM and calpain on NCX1.

Specific aims:

1. Investigate the role of PKA in NCX1 regulation and identify potential PKA phosphorylation site(s) (Paper I).
2. Investigate functional consequences and molecular mechanism of calpain cleavage of NCX1 and identify calpain cleavage site(s) (Paper II).
3. Investigate the function of the pSer68-PLM-NCX1 interaction by developing high affinity competitor peptides specific for the particular interaction (Paper III).
5. Methods

5.1 Human myocardial biopsies

In paper II, we aimed to investigate the molecular basis of calpain binding and cleavage of NCX1 in hypertrophic hearts with and without HF. In this study, we analysed small myocardial biopsies sampled from aortic stenosis (AS) patients, with pressure overload-induced hypertrophy, by Western blot analysis. Changes observed in such pathology are thought to be comparable to those observed in animal models of hypertrophy. Differences between hypertrophic left and hypertrophic right ventricles have been reported and analyses from Framingham Heart study reveal that patients with left ventricle hypertrophy (LVH) have a high risk for development of cardiovascular disease (134,135). Biopsies were therefore taken from LV because AS is associated with LVH. Several structural and functional changes associate with aortic stenosis (136,137). A recent study has reported several changes in cardiac proteins such as SERCA2a and myosin binding protein C in the LV from AS patients (138), supporting that LVH in AS is as a good model to study molecular changes of other ion handling proteins. The LV biopsies were taken from AS patients during elective aortic valve replacement (AVR).

Biopsies from patients with coronary artery disease (CAD) were used as negative controls. Notably, AS and CAD are two different pathologies; AS is an aortic valve disease (non-ischemic heart disease), while CAD is categorized as an ischemic heart disease. “Control samples” were therefore sampled from the non-ischemic area of cardiac tissue taken from the CAD patients during the coronary artery bypass graft operation (CABG).

5.2 Experimental animal models

In parallel with the human myocardial biopsy study (paper II), we used an ascending aortic banding rat model to demonstrate changes of protein levels during the development of LV hypertrophy and in the transition to HF. This model is well developed at our institute and is inexpensive. Moreover, the model is appealing as an experimental animal model because it provides high success in reproducibility of cardiac hypertrophy, low mortality rate after the surgical procedure, and greater amount of myocardial tissues available (compared to human and mice heart) for molecular and biochemical analyses (139-141).

The ascending aortic banded rat model is widely used in experimental research since its outcomes can be translated into clinical studies (142-144). However, there exist obvious differences between humans and rats. Experimental outcomes from animal studies do not
always reflect the actual progression of human heart diseases and these findings are therefore often not directly applicable in clinical level. First, heart rate (HR) of human and rat differs; 60-90 bpm and 250-600 bpm, respectively. Because rats have a faster HR, generation of its contractile force driven by SR Ca\(^{2+}\) cycling indicates a higher activity of SERCA2a pump in rat compared to human (145). Second, development of human heart diseases has several stages which are influenced by number of risk factors like hypertension, age, smoking, obesity and diabetes and the severity is progressed overtime. Unlike human HF, the experimental animals are exposed to the “pathogenic factor” such as coronary artery ligation leading to a relatively quick development of myocardial hypertrophy and HF. Nevertheless, the aortic-banded rat models have been shown to be useful to study cardiac biology in various pathophysiological stages, like hypertrophy and HF (142-144). In fact, the pressure overload manifested in aortic-banded rats resembles the progression of LVH leading to HF in human in the context of gradual deterioration (141). However, experimental manipulations in animal models such as ascending aortic banding and genetic models of hypertension and HF do not always completely reflect the alterations in human heart diseases. Despite the physiological and molecular settings differ in rats and humans, the common settings for ECC involved in Ca\(^{2+}\) handling in the heart are indeed similar. Such similarities allow researchers to explore signaling pathways responsible for both physiology and pathophysiology of the heart.

5.3 In vivo isoproterenol infusion

In paper I, the role of PKA phosphorylation of NCX1 was investigated by using isoproterenol (ISO) to activate β-adrenergic signaling in adult wild type rat hearts. The increase in adenosine 3′,5′-cyclic monophosphate (cAMP) levels following the exposure of the heart to isoproterenol results in PKA activation leading phosphorylation of several cardiac proteins (146,147). The approach is well-established at our institute and the procedure is highly reproducible for β-adrenergic stimulation in whole animal. Moreover, control animals were also included in the experiments by infusing the animals with a vehicle (0.9% NaCl) providing comparable data (i.e. level of phospho-proteins) between β-AR stimulation and non β-AR stimulation.

5.4 Culturing and isolation of cardiomyocytes

Cell cultures of neonatal and adult cardiomyocytes are widely used as in vitro system to study physiology and function of human cardiomyocytes (148). Adult cardiomyocytes are already differentiated. Their contractile properties and electrophysiological characteristics are
preserved in culture when the isolation procedure is optimal (evaluated by high yield of rod-shaped cardiomyocytes and the quality of each cardiomyocyte). Adult cardiomyocytes are difficult to isolate and cell yield and viability are usually not satisfactory after the isolation procedure. Therefore, primary cultures of neonatal cardiomyocytes were mostly chosen as an *in vitro* experimental model in paper I and paper II because of its several advantages.

Neonatal cardiomyocytes are easier to culture. In contrast to adult cardiomyocytes, neonatal cardiomyocytes grow efficiently and they have a great potential to differentiate in response to various stimuli (149). Moreover, several studies using culture of neonatal cardiomyocytes have identified important molecular mechanisms involved in regulation of cardiac hypertrophy *in vivo*, suggesting neonatal cardiomyocytes to be a good *in vitro* model to study hypertrophic responses (150,151). Several signaling mechanisms are reported to be similar in neonatal and adult cardiomyocytes, for example the effect of β-adrenergic stimulation (studied in paper I) (152,153).

In paper I, cultures of neonatal rat cardiomyocytes were used to study protein phosphorylation in response to different stimuli such as isoproterenol and forskolin which are thought to stimulate β-AR and activate PKA. In paper II, cultured neonatal cardiomyocytes were also stimulated by calcium in order to activate calpain and investigate the calpain cleavage of NCX1. Isolated membrane fractions from neonatal cardiomyocytes were used to study protein localization and protein-protein interaction.

Isolated adult rat cardiomyocytes were our choice to study NCX1-calpain 1 interaction by DUOLINK (Paper II) because of their morphology. In addition, they contain intact contractile units, distinct key proteins and protein posttranslational modifications (PMTs). PMTs are often important for enzymatic activity, protein interactions and subcellular location (154). Moreover, adult cardiomyocytes are extensively used for visualizing Ca²⁺ transients, contraction, signaling pathways and protein-protein interactions.

### 5.5 Cell culture of human embryonic kidney (HEK)

Transient transfection of the HEK293 cells was employed as a tool to express various proteins used in several investigations such as the mapping of protein-protein interaction sites, calpain cleavage of NCX1 *in vitro* and measurements of NCX1 activity. The major advantages of using the HEK293 cell line is a high yield of protein expression level with reproducible
results. HEK293 cells are easy to maintain, and offers a proper protein folding and a post-translational modification (PTM) machinery.

In paper II and III, transfected HEK293 cells were used for functional analyses of NCX1 using patch clamp experiments (described in section 5.7). Cell morphology of transfected HEK293 cells is an important criterion when recording whole-cell currents. Isolated transfected cells expressing green fluorescent reporter protein (GFP) as shown in Figure 8A were chosen for the recordings, whereas transfected cells in clusters (Figure 8B) were avoided due to formation of gap junctions which could interfere with the recordings of NCX1 currents.

**Figure 8** HEK293 cells expressing GFP. A. A viable transfected HEK293 cell suitable for patch–clamp experiment. B. Transfected HEK293 cells unsuitable for patch–clamp experiments. Cells are clustered and round–shaped. Figures are taken from Thomas 2005 (155).

### 5.6 General molecular biology techniques for protein-protein interaction studies

This thesis focuses on identification of various protein-protein interactions in the heart (paper I-III). A variety of molecular biology techniques has been employed to analyse protein binding such as various pull-down assays, co-immunoprecipitations (co-IPs), DUOLINK and peptide arrays.

#### 5.6.1 Co-IPs

Co-IPs are extensively used to identify potential indirect and direct protein interaction partners (Figure 9). An appropriate antibody (IP antibody) specific for the protein of interest (protein X) is added to the LV or cell lysate. The protein X bound antibody with the potential protein partner (protein Y) is then captured by a protein A/G matrix (beads). The immuno-complex, consisting of IP antibody - protein X - protein Y, is thereafter collected by
centrifugation, washed and analysed Western blot analysis. The potential protein partner is visualized by using an antibody specific for the appropriate protein. False positive interactions might arise when whole cell lysate containing mixed cellular compartments are used. To overcome this, cell lysate extracted from the specific subcellular compartment can be applied to demonstrate the particular protein interaction and hinder that two proteins from different cellular compartments interact in the cell lysate. False negatives can occur when too high stringent buffer is used in the washing steps (particular for weak and transient interactions) or when the protein interaction site overlaps with the antibody epitope (due to occupation of the protein partner at the antibody binding site). The primary antibody is then only able to bind to protein X without any protein Y bound. Thus, only protein X will be collected in the immunocomplex. The problem can be solved by exploiting exogenous proteins containing tagged molecule and using an IP antibody specific for the tagged molecule (as an external epitope). For endogenous proteins, another IP antibody (specific for another epitope) has to be used.

![Diagram](image.png)

**Figure 9** Both direct and indirect protein–protein interactions can be detected by Co–IPs. Briefly, an appropriate IP antibody specific for protein x (prot X) is added to the lysate. Protein partner Y (prot Y) can bind either directly or indirectly to protein X through a bridge protein Z (prot Z). Protein Y is identified by Western blot analysis.

### 5.6.2 Pull-down assays

Pull-down assay is an alternative approach to study protein-protein interactions. Pull down assay can be used to identify and analyse direct protein-protein interactions in various binding buffers. The method can be also used to validate interactions identified by co-IPs and other technologies such as peptide array overlay assays (described in section 5.6.4).

Pull-down assays exploit a variety of tagged molecules such as biotin, GST, FLAG or HIS in the “bait” protein. An affinity matrix specific for the affinity tag is used to capture the
complex (Figure 10). In this thesis, biotinylated peptides were extensively used as “bait” and anti-biotin agarose beads were used as an affinity matrix to immobilize the “bait”. The peptide bound beads is then incubated with an appropriate interacting partner (so-called “prey”). The direct binding of the protein partner to the biotinylated peptides is visualized by western blot analysis using specific antibody against the protein partner.

Figure 10 Schematic illustration of a pull-down assay. The affinity matrix (anti-biotin agarose beads) captures the biotin tagged peptide-protein complex. Binding of the protein partner will be visualized by Western blot analysis.

5.6.3 DUOLINK

Some of the limitations of pull-down and Co-IP assays mentioned above can be overcome by the DUOLINK in situ proximity ligation assay (PLA) technology. This technique was used to show the co-localization of NCX1 and calpain in adult cardiomyocytes (Paper II). DUOLINK allows identification of both weak and transient interactions and the interactions are detected only when the two proteins of interest are located in close proximity (<40 nm). One of the most challenging tasks in a DUOLINK analyses is to find a specific primary antibody for the two protein partners. Importantly, the two primary antibodies must be from different species so that the PLA probes (secondary species-specific antibodies containing unique DNA strands) can recognize and bind to the primary antibody (Figure 11). If the two proteins are located within 40 nm range, the unique DNA strands in the PLA probes will generate circle templates which then will be amplified during the step so-called rolling circle amplification (RCA). The products from the RCA (replicates of DNA circle) will be translated into PLA signals by complementary fluorescent labeled oligonucleotide probes used in detection step (156). PLA signals will be observed as a distinct fluorescent dot representing the particular interaction of interest. However, false positive signals can occur, decent negative controls must be included. In paper II, two negative controls were performed by omitting the primary antibodies and
using a specific blocking peptide to block the primary antibody binding to one of the target proteins. If available, cardiomyocytes from the gene knockout animal can be used as an additional negative control. Moreover, false positives generated by unspecific binding of antibodies can also be easily eliminated by using cells from KO cardiomyocytes that lack one of the target proteins.

![Diagram of PLA signal formation](image)

**Figure 11** Generation of the PLA signal in DUOLINK assay. Recognition of the two target proteins by their specific primary antibody followed by binding of a specific secondary antibody, so called PLA probe, to each primary antibody molecule. The formation of PLA signal is observed as a fluorescent spot. The figure is edited from [www.olink.com/products-services/duolink/situ-pla-technology](http://www.olink.com/products-services/duolink/situ-pla-technology)

### 5.6.4 Peptide arrays and soluble peptides

In this thesis, we have used peptide arrays to identify PKA phosphorylation sites (paper I), calpain cleavage site (paper II), protein/peptide binding sites (Paper II-III) and map antibody epitopes (Paper I-III). The peptide technology allows us to identify the various sites down to the amino acids level. To develop high affinity PLM-NCX1 disruptor peptides (paper III), we used two dimensional peptide arrays where each residue in the native NCX1 peptide sequences were systemically substituted with every possible amino acid residue.

The particular protein of interest is synthesized as 20-mer overlapping peptides on a cellulose membrane (Figure 12) by FMOC-chemistry (synthesized by a core facility at University of Oslo). The peptide array is then incubated with the purified protein partner (or a biotinylated peptide containing the reciprocal binding site), active enzyme or antibody. The linear binding motifs are identified by Western blot analysis. The results from peptide array provide only information about relative affinities and represent only semi-quantitative data. The interpretation must be done with cautions because there are several factors that can influence the signal intensities of the spot for example, inhomogeneity of the array.
False positives can occur if the secondary antibody binds non-specifically to the membrane or if excessive amount of protein are used in the overlay assay. The “true positives” can be distinguished from false positives by choosing an optimal blocking reagent and performing a parallel control experiment where the protein of interest is omitted. Moreover, in order to discriminate false positives from true binding motifs, the positive sequences can be synthesized as biotinylated peptides in solution. The binding of the biotinylated peptides to the protein partner can be verified by pull-down assays.

False negatives can arise when the structure of proteins is an important factor for the particular protein-protein or antibody-protein interaction. The protein is synthesized linearly on the peptide array membrane and thus, might not represent as an optimal conformation for the interaction to occur. In addition, false negatives might arise if the interactions require a particular posttranslational modification such as phosphorylation, O-GlcNAcylation or palmitoylation. Introduction of important posttranslational modifications (if known) on the peptide arrays can be performed to overcome such pitfalls.

Furthermore, a weak binding or an absence of binding of the protein partner to the peptide array might also happen due to the different properties of the peptides (hydrophilic vs. hydrophobic), which cause different yield of peptide on the array (157,158).
We also used peptide membranes to screen for phosphorylation sites within a protein (paper I). The protein kinase assay was performed directly on the membrane and the potential phosphorylated sites were detected by using autoradiography or phosphorimaging.

5.7 Electrophysiological techniques

In order to analyse NCX1 function, electrophysiological recordings of NCX1 variants expressed in HEK293 cells was performed using the patch clamp technique in whole-cell configuration (Figure 13) (159). In paper II we investigated the effect of calpain cleavage on NCX1 current and in paper III regulation of NCX1 by PLM. The cells were plated on glass cover slips and transferred to a recording chamber 24-48 hours following transfection. The growth medium was removed and the cells were then buffered with an artificial recording solution optimized for monitoring NCX1 currents. The HEK293 cells were observed and checked for fluorescence in an inverted light microscope. Successful transfection was based on observation of GFP expression. Typically, we could detect GFP in about 30-50% of the cells.

During the whole-cell patch clamp experiments, a pipette resistance between 1 and 3 Mohm (M Ω) was used to assure minimum series resistance and allow sufficient dialysis of the cells. The patch pipette was filled with an artificial intracellular solution (sterile filtered with 0.22 μm pore size). In order to monitor the membrane potential and record ionic currents, the patch pipette was mounted to a head stage which was connected to an amplifier and through the analog-to-digital converter (ADC), which is important for sending digitized signals to the computer (for data analysis and recording). To register NCX1 currents (whole-cell currents), formation of gigaseal is very important for the patch-clamp recording. In brief, a tight contact between the glass pipette tip and the surface of cell membrane (ohmic contact) was formed by applying mild suction. Such ohmic contact is so-called gigaseal. To create electrical contact with the whole cell, a sub atmospheric pressure (vacuum) is created by applying suction. Because the cell membrane behaves like a resistor capacitor (RC) circuit (whereby the transient charging and discharging resembling a capacitor and resistor, is working in parallel with the electrical circuit), and such electrical properties of the cell membrane is allowing us to monitor the capacitance of the cell membrane. Importantly, the membrane capacitance is a parameter corresponding to the cell surface area as it depends on the thickness of phospholipid bilayer. Therefore, the membrane capacitance was used to normalize the currents in all experiments. Notably, the unit of capacitance is “farad”. For all
experiments we used voltage clamp meaning that we clamped the voltage while monitoring the current. Because the NCX1 is a slow exchanger carrying current in both direction a ramp protocol was created by gradually decreasing the potential from 120 to -100 mV. The current goes in the opposite direction of the Ca$^{2+}$ because three Na$^+$ ions are exchanged for one Ca$^{2+}$ ion. The forward mode is therefore defined as the current going into the cell (Ca$^{2+}$ moving out). NCX1 is blocked by Ni$^{2+}$ and the subtracted (Ni$^{2+}$ sensitive) current observed in the experiments are visualized in a current (I)/ voltage (V) plot following normalization to the cell surface (in pico farad).

![Whole-cell patch clamp recording](image)

**Figure 13** Whole-cell patch clamp recording. The tight seal between the glass pipette and the membrane is formed, followed by a mild suction rupturing a small piece of the membrane in order to achieve whole-cell recording. Grey boxes represent NCX1 proteins expressed in the plasma membrane.
6. Main results

Paper I: Full-length cardiac Na+/Ca2+ exchanger 1 protein is not phosphorylated by protein kinase A

- PKA phosphorylated threonine 731 in the linearized NCX1 protein sequence.
- Threonine 731 was inaccessible for PKA phosphorylation in full length, calpain- and caspase-3 cleaved NCX1 protein.

Paper II: Molecular basis of calpain cleavage and inactivation of the Na⁺ - Ca²⁺ exchanger 1 in heart failure

- Full-length NCX1 and a proteolytic NCX1 fragment of 75 kDa were increased in aortic stenosis patients and rats with HF.
- Calpain expression and proteolysis of PKC-α (a calpain substrate) were both increased in the diseased hearts.
- Calpain catalytic domain and calpain domain III bound to catenin-like domain (NCX1-CLD) and to the first Ca²⁺ binding domain (NCX1-CBD1) of NCX1, respectively.
- Methionine 369 in NCX1-CLD was identified as a calpain cleavage site.
- Engineering NCX1-Met369 into a tobacco etch virus (TEV) protease cleavage site revealed that specific cleavage at NCX1-Met369 reduced NCX1 activity (without calpain activation) in HEK293 cells.

Paper III: Development of a high affinity phospholemman binding peptide that reverses phosphorylated serine 68 phospholemman inhibition of the cardiac sodium-calcium exchanger 1

- Both PLM and pSer68-PLM bound directly to PASKT and QKHPD-containing sequences in the catenin-like domain of NCX1.
- Conversely, PASKT and QKHPD-containing sequences bound to directly to PLM (41-60).
- Optimization of an NCX1 derived peptide from the QKHPD region, enhanced pSer68-PLM binding eight-fold.
- The optimized peptide blocked PLM/pSer68-PLM binding to NCX1-PASKT and NCX1-QKHPD and reversed PLM (S68E) inhibition of NCX1 in HEK293 cells.
7. Discussion

7.1. PKA regulation of NCX1

Regulation of NCX1 activity by PKA phosphorylation has been extensively studied, but the results are conflicting. Several groups have reported that β-adrenergic stimulation activates NCX1 activity by using isoproterenol (ISO), forskolin or cAMP analogs (91,160-162) whereas other laboratories have failed to observe any enhancement of NCX1 current following β-adrenergic stimulation (163-166). Linn et al. and Ginsberg et al. demonstrated that 1) isoproterenol did not enhance NCX1 activity in guinea pig, mouse, rat and rabbit ventricular myocytes and 2) the effect of cAMP-activated Cl⁻ current activation (CFTR channels) accounted for the increase in NCX1 current in response to isoproterenol (165,166). The contamination of Cl⁻ currents influencing NCX1 measurement (Ni²⁺-sensitive current) has also been confirmed by using rabbit atrial myocytes which lack PKA-dependent Cl⁻ currents (167). The authors concluded that PKA stimulation does not up-regulate NCX1 activity in rabbit cardiomyocytes. However, isoproterenol stimulation has been shown to enhance NCX1 activity in larger animals e.g. pigs and dogs, suggesting that there might be species differences (168).

A different subcellular environment around NCX1 in different species might also explain the discrepancy for PKA regulation of NCX1. PKA activation enhanced activity of cardiac NCX1 expressed in Xenopus oocytes (91), but it failed to mediate its effect on NCX1 activity when the cardiac NCX1 was expressed in HEK293 cells (93). Interestingly, some in vitro studies have demonstrated that PKA can phosphorylate cardiac NCX1 in rats and pigs. However, no phosphorylation sites were identified (91,92,162). These findings led us to further investigate PKA phosphorylation of NCX1.

In paper I, we employed bioinformatics and peptide array technology to identify putative PKA phosphorylation sites in NCX1. First, human, rat and mouse NCX1 were screened for putative PKA phosphorylation sites by bioinformatics analyses. NCX1 was further synthesized as a linearized protein i.e. overlapping 20-mer peptides on membranes. By using this technology, every possible amino acid residue in NCX1 could be screened for PKA phosphorylation in vitro. Four of the predicted PKA phosphorylation sites in silico, residing in the cytoplasmic loop of NCX1, appeared to be only little-to-modestly phosphorylated by PKA in vitro. However, several additional strong PKA phosphorylation sites were identified.
Mutational analyses of the putative PKA phosphorylation sites demonstrated that only one PKA site; Thr731 in NCX1, was a novel PKA phosphorylation site *in vitro*.

To investigate whether Thr731 could be phosphorylated by PKA *in vivo*, a specific phospho-Thr731 antibody was generated. Infusion of isoproterenol in rats was performed to activate PKA. Despite, strong indication of phosphorylated phospholamban at serine 16 was observed in response to β-adrenergic stimulation, phospho-Thr731 was absent, demonstrating that Thr731 was not phosphorylated by PKA *in vivo* which was likely due to the inaccessibility of the site. Inaccessibility was also demonstrated by using incorporation of radiolabeled phosphate group from [gamma-32P] ATP into an NCX1-GFP full-length fusion protein. Investigation of additional mechanisms promoting phosphorylation of NCX1 by PKA was also performed by exposing NCX1-GFP fusion protein for calpain and caspase-3. Proteolysis of NCX1 by these two proteases did not facilitate PKA phosphorylation of NCX1. Conclusively, our data strongly suggested that the PKA site was neither accessible in full-length NCX1 nor calpain/caspase-3 digested NCX1.

Interestingly, when our PKA-NCX1 data was published in “The American Journal of Physiology - Cell Physiology” in 2011, a model of ATP-dependent regulation of NCX1 by PKA was proposed by Morad and coworkers in “Editorial Focus” (169). The model suggesting a functional arrangement where the α-catenin-like domain (CLD) and the critical serine/threonine residues involved in PKA phosphorylation of NCX1 are brought into close vicinity. The fourth α-helix of the hypothetical α_CAT structure is located downstream of the second Ca^{2+} binding domain (β2) (Figure 14). Such rearrangement is proposed to facilitate signal transduction from Ca^{2+} binding domains (CBD1 (β1) and CBD2 (β2)) to the transmembrane domains through CLD. In this NCX1 configuration, Ser388, which has been reported to important for cAMP-regulation, and Thr731 (our findings), might become accessible for PKA to mediate ATP-dependent regulation of NCX1. Though, several experiments are required in order to verify this hypothesis as stated by the authors.

Although, we concluded that NCX1 is not a direct target for PKA phosphorylation, we cannot exclude the possibility that NCX1-Thr731 is accessible for PKA phosphorylation under other circumstances.
Figure 14 A hypothetical model of PKA–dependent regulation of NCX1 proposed by Morad et al (169). In this model, Thr731 might be accessible for PKA phosphorylation. The important serine (S) and threonine (T) residues are in green circles. Three domains of NCX1 are illustrated, 1) the nine transmembrane segments, 2) the α–catenin–like domain (α\text{Cat}) including its unstructured linker sequences (S1, S2, A/B–CDEXF) as indicated in green along the cytoplasmic loop, and 3) the Ca$^{2+}$ binding domains with β–sandwich structures (β1 and β2). Ser722 and Thr723 were not found to be true PKA phosphorylation sites in our study (170). Figure is taken from Morad 2011.

The functional role of PKA on NCX1 activity might be indirect. Protein interaction partners of NCX1 might be directly phosphorylated by PKA, and thus, regulating NCX1 activity. Several cardiac proteins are PKA substrates, for instance phospholemman (PLM), a robust PKA and PKC substrate, which has been identified as an endogenous regulator of NCX (171,172). Phosphorylation of PLM at serine 68 (either by PKA or PKC) leads to NCX1 inhibition (104,173) but enhances NKA activity (174,175). Constitutive overexpression of phosphorylated phospholemman (S68E, mimicking phosphorylation of serine 68) has been shown to result in NCX inhibition and animals with arrhythmias and HF (116).

7.2. Insight into the functional significance of calpain-cleaved NCX1

In paper II, we investigated the regulatory role of calpain on NCX1 activity. Calpain activation can be induced by various pathological stimuli such as oxidative stress and Ca$^{2+}$ overload and is shown to be involved in cardiac remodeling and HF (128). Unlike digestive
proteases such as proteasomes, calpains are Ca\(^{2+}\)-activated proteases that mediate limited proteolysis, resulting in altered function of the substrates (176). For example, calpain cleavage of protein kinase C has been shown to result in a constitutive active form of PKC (177).

Consistent with previous studies (178-180), we observed a significantly increase in protein expression levels of NCX1 and calpain in AS patients and rats with HF (paper II). Concomitantly, we found an increased level of a 75-kDa proteolytic NCX1 fragment in parallel with an increased level of PKC fragmentation, suggesting that calpain was activated in the diseased hearts.

Calpain-mediated proteolysis of different NCX isoforms has also been reported in brain, endoplasmic reticulum, caveolae vesicles, cardiac muscles and mitochondria (127,129,130,170,181). Although calpain-mediated cleavage of NCX1 has been demonstrated to result in constitutive activation of NCX1 (131), cleavage of NCX3 by calpain during brain ischemia has been shown to inhibit NCX3 activity leading to Ca\(^{2+}\) overload (127). Four potential calpain cleavage sites were identified in NCX3 and these cleavage sites resulted in three NCX3 fragments. Interestingly, three of these cleavage sites resulted in 58-60 kDa NCX3 fragment and they were located within a region that is highly varied between NCX1, NCX2 and NCX3. The fourth potential cleavage site was identified at lysine in position 370 (Lys370) in NCX3 which is corresponding to Lys377 in NCX1. However, this cleavage site was shown to be inaccessible in all three NCX isoforms in vivo. Consistent with the finding from Bano et al, we could not observe any calpain binding to Lys377 in the peptide overlay assays (182). Overexpression of calpastatin in neurons has been shown to prevent Ca\(^{2+}\) overload (127).

Interestingly, we found that calpain domain IIb and domain III interacted directly with CLD and CBD1 of NCX1, respectively (paper II). Domain IIb is the calpain catalytic domain consisting of the active site important for cleavage, and domain III is important for Ca\(^{2+}\) binding and membrane targeting (183). Using online software, several calpain cleavage sites were predicted within the cytoplasmic part of NCX1. We were particular interested in calpain cleavage sites that could theoretically give a 75-kDa NCX1 fragment. The prediction of calpain cleavage sites was initially performed by using online software based on Support Vector Machine method (SVM) and the prediction quality of SVM method was thought to be 76.86% (184,185). Notably, a methionine at position 369 in the cardiac NCX1 came up with
the highest score. Another software program using group-based prediction system (GPS algorithm) with over 80% prediction performance also suggested Met369 to be one of potential calpain cleavage sites (186). Consistently, we found that cardiac NCX1 was cleaved by calpain at the methionine 369 (NCX1-Met369) in vitro. In vivo cleavage was confirmed by using a specific custom made NCX1-Met369 antibody. NCX1-Met369 was located within the catenin-like domain (CLD), which is an unstructured part of NCX1 (38). Finally, a peptide docking model showed that the short NCX1-Met369 peptide fitted well into the active cleft between domain IIa and IIb in the human calpain (182).

In order to determine the direct effect of cleavage of NCX1 at Met369, we mutated the calpain cleavage site into a Tobacco etch virus (TEV) cleavage site. The introduction of a TEV cleavage site at Met369 allowed us to specifically cleave NCX1 without calpain activation. Specific cleavage of NCX1 at Met369 resulted in reduction of NCX1 activity (without calpain activation), suggesting that cleavage of NCX1 at Met369 leads to inactivation of NCX1. In this way, we could exclude any indirect effect of calpain on NCX1 through other regulatory proteins.

Contrary to our findings, calpain was shown to constitutively activate cardiac NCX1 expressed in Xenopus oocytes (131). Calpain can have indirect effect on NCX1 activity by cleaving and activating other regulatory proteins of NCX1. PKC is identified as a part of NCX1 macromolecular complex (92). Iwamoto et al have demonstrated that PKC phosphorylation of NCX1 increases NCX1 activity (187,188). NCX1 can be phosphorylated by PKC at three residues, serine 249, serine 250 and serine 357, where serine 250 is the major PKC site (188). Although alanine substitutions of all three PKC sites abolished PKC phosphorylation of NCX1, the substitutions did not have a significant effect on NCX1 activity. These results suggested that regulation of NCX1 by PKC might not require direct phosphorylation of NCX1 (188). Interestingly, calpain cleavage of PKC has been reported to give a constitutive active C-terminal PKC-α fragment, where the requirement of its cofactors such as phospholipids and phorbol ester is compromised (189,190). Calpain cleavage leading to a constitutive active PKC has been reported to be responsible for development of cardiomyopathy in mice (190). It is possible that calpain-activated PKC increases NCX1 activity in various pathophysiological conditions. Thus, calpain might regulate NCX1 activity through both PKC activation and limited proteolysis of NCX1, resulting in positive and negative regulation, respectively.
Whether the calpain-induced cleavage of NCX1 is beneficial or detrimental for the heart, remains to be clarified.

NCX1 has been shown to exist as an NCX1-ankyrin-B-NKA macromolecular complex (76,191). Interestingly, calpain has also been shown to degrade ankyrin and α- fodrin (key proteins for membrane-cytoskeleton complex) in addition to NKA. Proteolysis of ankyrin might affect NCX1 activity by disrupting the association between NCX1 and NKA (192). Moreover, any decrease in ankyrin function might also result in reduction in the levels of NCX1 and NKA (191,193,194). Both NKA α1- and NKA α2- isoforms are expressed in cardiomyocytes, where the latter isoform has been shown to modulate NCX1 activity (73). In vitro cleavage analyses using recombinant calpain showed that the NKA α2 isoform was more sensitive to calpain compared to NKA α1 (192,195). These results indicate an additional role of calpain by regulating NCX1 activity indirectly.

Several studies have reported that calpain inhibition is cardioprotective in acute pressure overload (196,197), and cardiac ischemia/reperfusion (198-200). Moreover, calpastatin (calpain inhibitor) transgenic mice have also been reported to prevent left ventricular hypertrophy induced by angiotensin II (201). In line with a cardioprotective role of calpain inhibition, recent studies on diabetes-associated cardiovascular complications show that disruption of calpain function by using cardiac specific calpain knockout mice (202) or calpain inhibitors (203) resulted in improvement of cardiac function.

Although several evidences confirm the implication of calpain activation in cardiac dysfunction, its molecular roles in pathophysiology remain to be determined. Nuclear factors of activated T cells (NFAT) and nuclear factor-κB (NF-κB) play both critical roles in cardiac hypertrophy and ventricular remodeling (204). Calpain cleavage of calcineurin at the autoinhibitory domain activates the phosphatase constitutively, leading to dephosphorylation of pNFAT and translocation of NFAT to the nucleus. Nuclear NFAT then triggers hypertrophic gene expressions (205-207). Calpain is also involved in activation of the NF-κB signaling pathways (208) which further contribute to angiotensin II-dependent left ventricular hypertrophy (201). Thus, calpain modulates many intracellular signaling pathways involved in cardiac remodeling.

Homozogous deletion of calpain 4/CAPNS1, the small subunits of both μ- and m-calpains (encoded by Capns1 gene), causes loss of protein expression and activity of both μ- and m- calpains resulting in embryonic lethality (209), suggesting that calpain also has
important physiological functions. Calpain has been shown to play a cardioprotective role by facilitating protein turnover and plasma membrane repair (210,211). Under normal physiological conditions, cardiac-specific calpain 4-deficient mice exhibited normal cardiac function, but were prone to cardiac dysfunction and membrane damage in response to haemodynamic stress such as pressure overload and β-adrenergic stimulation (211). Finally, calpain-1 appears to have higher activity and sensitivity to Ca$^{2+}$ compared to calpain-2 under basal conditions, supporting the physiological role of calpain-1 in the heart. Aberrant increased activity of calpain-1 is associated with pathological conditions (183,210).

7.3 NCX1 as a therapeutic target treating heart failure

Ca$^{2+}$ homeostasis plays an essential role in cardiac function. In particular, Ca$^{2+}$ removal mechanisms is altered during HF. NCX1 is a major mechanism for Ca$^{2+}$ removal and its transport rate is regulated by membrane potential and Na$^+$ and Ca$^{2+}$ concentrations. The role of NCX1 in Ca$^{2+}$ removal becomes a prominent mechanism during HF, indicating that changes in NCX1 function is a compensatory mechanism (62). As other Ca$^{2+}$ handling proteins such as SERCA2a, are reported to be down-regulated in failing heart, NCX1 is believed to maintain normal cardiac contractility either by improving systolic or diastolic function (212,213). However, the compensatory role of NCX1 might also lead to cardiac dysfunction (as discussed below), and the regulatory mechanisms responsible for the transition from a normal to pathophysiologic state are therefore important for development of therapies targeting NCX1. Regarding inhibition of NCX1 by calpain cleavage (paper II), our findings might shed some light on a compensatory role of NCX1 regulated by calpain during pathophysiological conditions in order to improve systolic function. However, inactivation of NCX1 by calpain can be detrimental for diastolic function.

Alterations of NCX1 possibly causing systolic dysfunction (due to depletion of SR Ca$^{2+}$) is shown in figure 15A. When SERCA2a activity is reduced, in association with excessive SR Ca$^{2+}$ release through hyperphosphorylated RYR2 (leaky RyR), forward mode NCX1 activity can potentially contribute to depletion of SR Ca$^{2+}$ and thus impair systolic function (85,214,215). In such situation inhibition of forward mode NCX1 might be favorable only in combination with enhanced SERCA2a function (216).

On the other scenario, changes in NCX1 could improve systolic function. In HF, when NKA activity is reduced, accumulation of [Na$^+$]$_i$ favors Ca$^{2+}$ influx via NCX1 (reverse mode) and/or reduce Ca$^{2+}$ efflux (forward mode). In combination with preserved SERCA2a function,
the increased Ca$^{2+}$ influx via reverse mode NCX1 can improve systolic function. However, too high rate of Ca$^{2+}$ influx mediated by NCX1 is harmful to the heart because Ca$^{2+}$ overload can follow and lead to contractile dysfunction and arrhythmias (51) (Figure 15B). Few potent NCX1 blockers, suppressing reverse mode of NCX1 have been developed and they have been shown to have anti-arrhythmic effect and prevent Ca$^{2+}$ overload (217). However, further experiments are needed to improve their selectivity/specificity. Control of cardiac contractility is manifested by several mechanisms, therefore, a combination of NCX1 inhibition and/or relief of inhibition with additional drugs targeting other Ca$^{2+}$ handling systems, might be of great interest (218). Therefore, manipulation of NCX1 activity in order to improve cardiac contractility requires better understanding of its regulation regarding to which type of HF patient is suffering from (diastolic dysfunction or systolic dysfunction).
Figure 15 Disruption of EC–coupling by increased NCX1 activity. A: SR Ca$^{2+}$ depletion can occur when three Ca$^{2+}$ ion handling mechanisms are altered; 1) NCX is upregulated (forward mode), 2) RYR2 are hyperphosphorylated, leading to high Ca$^{2+}$ leakage and 3) SERCA2a is down-regulated (low Ca$^{2+}$ re–uptake back to SR). B: Ca$^{2+}$ overload can occur when Ca$^{2+}$ fluxes are imbalanced and NCX1 operates mostly by reverse mode or reduced forward mode because of accumulation of [Na+]$_i$, resulting in Ca$^{2+}$ loading (unchanged function of SERCA2a and RYR2). CSQ; calsequestrin, PLB; phospholamban, RyR2; RyR, SERCA2a; SERCA. Figure is modified from Louch 2012 (219).
7.4. Development of a peptide inhibitor specific for the pSer68-PLM-NCX1 interaction

Phospholemman (PLM) has been identified as an endogenous inhibitor of NCX1 but its direct regulation on NCX1 activity has been controversial. The NCX1-PLM interaction has been observed in adult rat myocytes (220), guinea pig cardiac myocytes (109) and in heterogeneous protein expression system (172). However, other studies have reported an absence of the NCX1-PLM interaction by fluorescence resonance energy transfer (FRET (112) as well as by IPs (221). Moreover, their overall conclusions also suggest that the alteration in NCX1 activity in response to β-AR stimulation is a consequence of direct effect of PLM on NKA rather than direct regulation of NCX1 by pSer68-PLM (113,114).

In paper III, we aimed to investigate the biological functional of the pSer68-PLM-NCX1 interaction by developing inhibitor peptides. Consistent with previous studies (172,222,223), we confirmed that PLM bound to PASKT- and QKHPD-containing sequences in the cytoplasmic loop of NCX1 by using peptide array technology. Both PASKT and QKHPD motifs are important for regulation of NCX1 activity (224). Reciprocal binding of PASKT- and QKHPD-motifs was lying within amino acids 41-60 in the cytoplasmic tail of PLM, suggesting that each motif bound to each PLM monomer (Paper III).

To develop specific inhibitor peptides, PLM binding sites were mapped in the cytoplasmic loop of NCX1 by using peptide array technology. Several PLM binding sequences were identified, and four peptide sequences encompassing the two motifs; PASKT and QKHPD, were chosen for further analysis. All four peptide sequences bound to PLM with similar affinity, suggesting that all of them were potential candidate peptides for further development. However, two of the peptide sequences were excluded, because they partially encompassed the autoinhibitory region of NCX1 (XIP region) (33). The two remainder peptide sequences, containing the QKHPD motif, were chosen for further optimization. Alanine substitutions into the QKHPD motif have been shown to abolish pSer68-PLM inhibition of NCX1, and the lysine (K) within QKHPD is crucial for inhibitory effect of pSer68-PLM on NCX1 (224). Alanine mutation into the PASKT motif has also been shown to reduce the NCX1-PLM interaction and to abolish the inhibition of NCX1 by pSer68-PLM (224).
By using two-dimensional peptide array technology, every amino acid in the peptide sequence could be substituted with the other nineteen amino acids, allowing us to identify which amino acid substitution in which position that had the highest affinity for pSer68-PLM. A total of 380 peptides were screened. The most promising mutations were further combined in attempt to increase the pSer68-PLM affinity to an even higher level. It was only possible to optimize the peptide containing the partial QKHPD motif; 1-KHPDKEIEQLIELANYQVLS-20. We found that substitution of K (lysine) and D (aspartic acid) with two Y (tyrosine)); 1-YHPYKEIEQLIELANYQVLS-20, enhanced the binding affinity to pSer68-PLM eight–fold. This optimized peptide efficiently blocked NCX1-PASKT and NCX1-QKHPD binding to PLM/pSer68-PLM in overlay assays. The optimized peptide reversed the inhibitory effect of PLM (S68D) on NCX1 activity in HEK293 cells. Altogether, our findings confirmed the NCX1-PLM interaction and direct regulation of NCX1 by pSer68-PLM.

Consistent with our finding, recent study has shown that constitutive overexpression of S68E (mimicking phosphorylation at serine 68) in mice is damaging the heart, causing arrhythmogenesis, early mortality and HF (116). In addition, the inhibitory effect of pSer68-PLM on NCX1 has also been shown to be specific to NCX1 without influence on NKA activity (115,225). The optimized peptide described above might be beneficial to manipulate NCX1 activity in different pathophysiologic states.

Further experiments are required to determine whether the optimized peptide disrupts the pSer68-PLM-NCX1 interaction or induces conformational changes of pSer68-PLM, contributing to the relief of its inhibitory effect on NCX1 activity. PLM interacts also with NKA, but only through a transmembrane domain (226). Thus, it is less likely that the peptide interfere with the NKA-PLM interaction. PLM interacts also with LTCC, however, the reciprocal binding sites have not been described (109). Further studies are required to demonstrate whether the effect of the optimized peptide in cardiomyocytes coincides with its effect in HEK293 cells.

In order to investigate the biological function of the optimized peptide in vivo, the peptide must be optimized further to improve its stability and half–life properties. Peptides are in general instable and have short half-life due to degradation by peptidases circulating in animals and human (227,228). Several strategies to develop peptide analogs in order to improve the stability, sustain the biological activity and increase permeability have been
described in the literature (229). The peptide, or alternative a small molecule, need also tissue specific delivery. However, these topics were out of the scope of this thesis.

7.5. Is PLM-NCX1 interaction a potential drug target in heart failure?

PLM is a cardiac stress protein (111). Upon β-adrenergic activation, PLM gets phosphorylated and regulates both NKA and NCX1 activity in order to sustain normal contractility and prevent arrhythmogenesis. Phosphorylation of PLM results in NKA activation and a decrease in [Na\(^+\)]\(_i\) (100,102,230). Lower [Na\(^+\)]\(_i\) promotes Ca\(^{2+}\) efflux via NCX1. Such increase in Ca\(^{2+}\) efflux via NCX1 reduces the risk of arrhythmogenesis but reduce contractility. To maintain normal contractility, pSer68-PLM inhibits NCX1 activity by increasing diastolic Ca\(^{2+}\) and thereby increasing SR Ca\(^{2+}\) content (173,225). Thus, PLM is an important regulator to balance both [Na\(^+\)]\(_i\) and [Ca\(^{2+}\)]\(_i\) in the cell during stress conditions. The pSer68-PLM level is reported to be increased in both rabbit and human HF (174), suggesting inhibition of NCX1 in HF, a situation which can lead to imbalance of [Ca\(^{2+}\)]\(_i\).

In HF, the action potential is prolonged and excitation-contraction coupling (ECC) is disrupted(231). The prolonged action potential leads to impairment of relaxation (diastolic dysfunction) due to a prolonged reverse mode or delayed forward mode of NCX1 in response to the high [Na\(^+\)]\(_i\) in the failing heart (232). Acceleration of Ca\(^{2+}\) efflux via NCX1 is thought to improve relaxation and thus, relief of the inhibitory effect of pSer68-PLM on NCX1 activity in forward mode might be a possibility to increase Ca\(^{2+}\) removal and further improve diastolic function.

During depolarization, activation of NCX1 (forward mode) in association with Na\(^+\) entry (via voltage-gated sodium channel), increases [Na\(^+\)]\(_i\). Elevation of [Na\(^+\)]\(_i\), together with the depolarization shifts the operation mode of NCX1 toward Ca\(^{2+}\) entry via its reverse mode. Reverse mode of NCX1 has been suggested to trigger CICR (39,40), which is an important process for cardiac contractility. However the role of reverse mode NCX1 in CICR has been a controversial subject. Some studies observed low efficiency of reverse mode NCX1 in regulating CICR (47,233), and hence the ability of reverse mode NCX1 to trigger Ca\(^{2+}\) release appeared to occur under artificial conditions (234,235). Recent studies by Larbig et al and Torres et al demonstrated that reverse mode NCX1 exerts a synergistic mechanism together with activation of LTCC by priming the dyadic cleft with Ca\(^{2+}\), contributing to efficient Ca\(^{2+}\) release from SR (236-238). In this context, the inhibition of NCX1 by pSer68-PLM can perhaps hinder the regulatory function of NCX1 in CICR. Thus, relief of the inhibitory effect
of pSer68-PLM on NCX1 activity might be beneficial in attempt to increase the coupling efficiency of CICR.

Interestingly, the optimized pSer68-PLM binding peptide (paper III) was shown to increase both forward and reverse mode of NCX, suggesting it might be a potential pro-drug to improve both diastolic and systolic function in HF. However, the specificity and efficiency of the optimized peptide must be examined and validated, as several pharmacological drugs targeting NCX1 appear to influence other Ca\textsuperscript{2+} ion handling systems (217,239).
8. Conclusions

1. A novel in vitro PKA phosphorylation site was identified at Thr731 in the cytoplasmic loop of NCX1. The PKA phosphorylation site was not accessible in full-length NCX1 protein or calpain or caspase-3 digested NCX1, strongly indicating that NCX1 is not a direct target for PKA.

2. Full length NCX1 and a 75 kDa proteolytic NCX1 fragment was increased in human aorta stenosis patients and in rats with HF. Methionine 369 (Met369) in the cytoplasmic loop of NCX1 was identified as a calpain cleavage site. Cleavage at NCX1-Met369 leads to a 75 kDa proteolytic NCX1 fragment and inhibition of NCX1 activity in HEK293 cells.

3. PASKT and QKHPD containing sequences in NCX1 bound directly to PLM (41-60) in vitro.

4. An NCX1 derived peptide with high affinity for pSer68-PLM was developed and shown to be a potent blocking peptide for the pSer68-PLM-NCX1 interaction.

5. The optimized peptide relived the inhibitory PLM (S68D) effect on NCX1 in HEK293, thus confirming the direct regulatory role of pSer68-PLM on NCX1 activity.
9. References


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10. Appendix: Paper I-III
Molecular Basis of Calpain Cleavage and Inactivation of the Sodium-Calcium Exchanger 1 in Heart Failure*

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Background: Sodium-calcium exchanger 1 (NCX1) and calpain are up-regulated in heart failure (HF). Molecular mechanisms and functional consequences of NCX1 cleavage by calpain are not known.

Results: Calpain anchors to two NCX1 regions and cleaves at methionine-369, leading to inactivation.

Conclusions: Calpain inhibition might improve cardiac function.

Significance: Calpain might play a pivotal role in NCX1 regulation during HF.

Cardiac sodium (Na\(^+\))-calcium (Ca\(^{2+}\)) exchanger 1 (NCX1) is central to the maintenance of normal Ca\(^{2+}\) homeostasis and contraction. Studies indicate that the Ca\(^{2+}\)-activated protease calpain cleaves NCX1. We hypothesized that calpain is an important regulator of NCX1 in response to pressure overload and aimed to identify molecular mechanisms and functional consequences of calpain binding and cleavage of NCX1 in the heart. NCX1 full-length protein and a 75-kDa NCX1 fragment along with calpain were up-regulated in aortic stenosis patients and rats with heart failure. Patients with coronary artery disease and sham-operated rats were used as controls. Calpain co-localized, co-fractionated, and co-immunoprecipitated with NCX1 in rat cardiomyocytes and left ventricle lysate. Immunoprecipitations, pull-down experiments, and extensive use of peptide arrays indicated that calpain domain III anchored to the first Ca\(^{2+}\) binding domain in NCX1, whereas the calpain catalytic region bound to the catenin-like domain in NCX1. The use of bioinformatics, mutational analyses, a substrate competitor peptide, and a specific NCX1-Met369 antibody identified a novel calpain cleavage site at Met369. Engineering NCX1-Met369 into a tobacco etch virus protease cleavage site revealed that specific cleavage at Met369 inhibited NCX1 activity (both forward and reverse mode). Finally, a short peptide fragment containing the NCX1-Met369 cleavage site was modeled into the narrow active cleft of human calpain. Inhibition of NCX1 activity, such as we have observed here following calpain-induced NCX1 cleavage, might be beneficial in pathophysiological conditions where increased NCX1 activity contributes to cardiac dysfunction.

The cardiac Na\(^+\)/Ca\(^{2+}\) exchanger 1 (NCX1)3 is a central component in maintaining normal [Ca\(^{2+}\)]\(_i\), homeostasis and cardiac function. NCX1 is a bidirectional transporter that mediates the exchange of three Na\(^+\) for one Ca\(^{2+}\) across the plasma membrane in either forward mode (Ca\(^{2+}\) influx) or reverse mode (Ca\(^{2+}\) efflux) (1, 2). The direction of ion transport depends on the membrane potential and the intracellular and extracellular concentrations of Ca\(^{2+}\) and Na\(^+\). Under physiologic conditions, NCX1 functions predominantly as a Ca\(^{2+}\) extrusion mechanism, and the contribution to decline of the Ca\(^{2+}\) transient varies from 9 to 30%, dependent on species (3). In pathological settings, the reverse mode of NCX1 function is often augmented (4, 5).

Increased NCX1 expression and/or activity have been linked to disrupted Ca\(^{2+}\) homeostasis during hypertrophy, ischemia/reperfusion, arrhythmia, and heart failure (HF) (4, 5). Interestingly, in animal models of myocardial infarction, increased NCX1 activity was accomplished by only a modest increase in the NCX1 protein level, indicating that other regulators of the exchanger are also involved (6). Mammalian NCX1 consists of nine transmembrane segments (TMs). The large intracellular loop between TM5 and TM6 consists of ~500 amino acids, containing two Ca\(^{2+}\) binding regulatory domains, CBD1 and CBD2 (7).

3 The abbreviations used are: NCX, Na\(^+\)/Ca\(^{2+}\) exchanger; HF, heart failure; LV, left ventricle; FL, full-length; TM, transmembrane segment; CBD, Ca\(^{2+}\) binding regulatory domain; CLD, catenin-like domain; XIP, exchanger inhibitory peptide; AS, aortic stenosis; ABHT, aortic banding hypertrophy; ABHF, aortic banding heart failure; HEK, human embryonic kidney; TEV, tobacco etch virus; CaMPDB, Calpain for Modulatory Proteolysis Database; ER, endoplasmic reticulum; SERCA, sarcoplasmic endoplasmic reticulum Ca\(^{2+}\)-ATPase; SR, sarcoplasmic reticulum; PLA, proximity ligation assay.
Ca\(^{2+}\) also activates a variety of Ca\(^{2+}\)-dependent signaling molecules, including the ubiquitously expressed, non-lyosomal cysteine protease calpain (8). Calpain is a cytoplasmic heterodimer composed of a catalytic subunit (80 kDa) and a regulatory subunit (30 kDa). There are two major catalytic isoforms, calpain-1 and calpain-2, which are activated by micromolar and nearly millimolar Ca\(^{2+}\) concentrations, respectively. Active calpain cleaves its substrate with a limited specificity, suggesting that it is a regulatory protease. Calpain is implicated in various pathological conditions associated with Ca\(^{2+}\) overload (9–12); however, little is known of the precise molecular mechanisms and biological consequences of calpain-dependent cleavage of proteins. Interestingly, the ubiquitously expressed NCX1 has been shown to be cleaved into a proteolytic fragment of ∼75-kDa in various tissues (9, 13, 14).

In the present study, we hypothesized that calpain is an important regulator of NCX1 in response to pressure overload. We aimed to identify the molecular mechanisms and functional consequences of calpain binding and cleavage of NCX1 in normal heart and HF.

**EXPERIMENTAL PROCEDURES**

**Human Left Ventricle (LV) Biopsies**—The human myocardial biopsy protocol conformed to the Declaration of Helsinki and was approved by the Regional Committee for Research Ethics in Eastern Norway (Project 2010/2226). Informed written consent was obtained from all patients. LV apical myocardial biopsies (5–10 mg) were obtained immediately before cross-clamping the aorta in eight patients undergoing elective aortic valve replacement for severe aortic stenosis (AS). All patients exhibited preserved ejection fraction (>50%) and no significant coronary artery stenosis. Myocardial biopsies were also obtained from eight patients undergoing elective coronary artery bypass graft (CABG) surgery, which served as controls. This patient group exhibited ejection fraction >50%, stable angina pectoris, and no evidence of peri-operative ischemia, previous myocardial infarction, or significant valvular disease. If left ventriculography was normal, echocardiography was not performed in control patients, in accordance with hospital guidelines. The included patients were receiving the following cardiovascular medications (aortic valve replacement/control): salicylates (2/7), warfarin (2/1), angiotensin-converting enzyme inhibitors (4/3), Ca\(^{2+}\) antagonists (2/1), cholesterol-lowering medication (3/8), diuretics (2/0), and β-blockers (2/3). Biopsies were taken from normal appearing and contracting regions of the myocardium using a 16G MaxCore disposable biopsy instrument (Bard, Tempe, AZ), snap-frozen in liquid nitrogen, and stored at −70 °C.

**Animal Model**—Animal handling and experiments were approved by the Norwegian Animal Research Committee (FOTS ID 3820) and conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication 85-23, revised 1996). Male Wistar rats (Møllergaard Breeding and Research Center, Skensved, Denmark) weighing ∼170 g were subjected to aortic banding as described previously (15, 16). In short, anesthesia was induced in an anesthesia chamber containing a mixture of 67% N\(_2\)O, 28% O\(_2\), and 4% isoflurane. Ventilation was performed by subsequent endotracheal intubation with a respirator (Zoovent, Triumph Technical Services, Milton Keynes, UK), and anesthesia was maintained by administration of a mixture of 69% N\(_2\)O, 29% O\(_2\), and 2% isoflurane. The chest was opened in the left, second intercostal space, and the ascending aorta was carefully dissected. A significant stenosis was induced by a tight banding of the ascending aorta with a 3.0 silk suture. For sham-operated animals, the silk suture around the ascending aorta was not tightened. Buprenorphine was administered for postoperative analgesia. After 6 weeks, echocardiography was performed with a Vevo 2100 (Fujifilm VisualSonics, Canada), and short and long axis images of the LV and atrium were obtained. Flow through the mitral and aortic valve was measured. Classification of aortic banding animals into hypertrophy (ABHT) or congestive HF (ABHF) groups was determined by echocardiographic, hemodynamic, and post-mortem analyses. Criteria for inclusion in the ABHT group were increased posterior wall diameter (>1.9 mm), increased LV weight (>0.75 g), and preserved lung weight (<2.0 g). The criteria for inclusion in the ABHF group were the same as for ABHT, except with increased lung weight (>2.5 g) and left atrial diameter >5.0 mm (16). The S group served as a control. All LV samples were snap-frozen in liquid nitrogen and stored at −70 °C until analyses.

**Primary Cultures of Neonatal Rat Cardiomyocytes**—Cultures of rat neonatal cardiomyocytes were prepared from the LV of 1–3-day-old Wistar rats. The LV was excised, minced, and enzymatically digested with collagenase solution. The cell suspension was incubated in uncoated culture flasks in a humidified incubator with 5% CO\(_2\) at 37 °C for 20 min. After 20 min, the non-adherent cells were isolated as cardiomyocytes and seeded onto 6-well culture plates (precoated with 0.2% gelatin) at a density of 3.75 × 10\(^{2}\) cells/mL in plating medium consisting of DMEM (D1152, Sigma-Aldrich), M-199 (M2520, Sigma-Aldrich), penicillin/streptomycin (P0781, Sigma-Aldrich), horse serum (14-403E, BioWhittaker, Walkersville, MD), and fetal bovine serum (FBS) (14-701F, BioWhittaker). The cells were incubated in a humidified incubator with 5% CO\(_2\) at 37 °C for 24 h before protein fractionation.

**Adult Cardiomyocyte Isolation**—Rats were anesthetized in a chamber filled with 95% room air and 5% isoflurane (Abbott Scandinavia Ab, Solna, Sweden). Cervical dislocation was performed after abolished pain reflexes were verified. Hearts were then quickly excised and placed in cold 0.15 M NaCl solution with heparin (Heparin LEO, 5000 IE/ml; Orifarm AS, Oslo, Norway). The aorta was then cannulated and retrogradely perfused with a cell isolation buffer containing 130 mM NaCl, 25 mM Hepes, 22 mM D-glucose, 5.4 mM KCl, 0.5 mM MgCl\(_2\), 0.4 mM Na\(_2\)HPO\(_4\), (pH 7.4) (all from Sigma) to wash out blood. The heart was thereafter perfused with cell isolation buffer containing 200 units ml\(^{-1}\) collagenase type II (Worthington) and 0.1 mM Ca\(^{2+}\). After a 20-min perfusion, the heart was cut down, and the atria and right ventricle were removed. The LV was minced and gently shaken at 37 °C for 3–4 min in the same solution used in the perfusion but with the addition of 1% BSA and 0.02 units/ml deoxyribonuclease I (Worthington). The digested ventricular tissue was then filtered (200-μm nylon
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mesh), and cardiomyocytes were sedimented. The cardiomyocyte pellet was resuspended in cell isolation buffer with 1% BSA and 0.1 M CaCl₂ solution. Isolated cardiomyocytes were stored at room temperature until use.

Antibodies—Anti-NCX1 (custom-made, epitope: GQPVRKFVKHARDHPIZIPST), anti-Met₃⁶⁹-NCX1 (custom-made, epitope: TRLMTGAGNILKRH), and anti-δ-His were obtained from Genscript Corp. (Piscataway, NJ). Anti-calpain-1 (H-240) (sc-30064), anti-PKC-α (C-20) (sc-280), anti-EFGR (1005) (sc-03), and anti-GAPDH (V-18) (sc-20357) were from Santa Cruz Biotechnology, Inc. Anti-calpain-1 (ab39170 and ab89778) were from Abcam (Cambridge, MA), and anti-calpain-1 domain III (9A4H8D3) (208728) was from Calbiochem. Anti-GFP (Living Colors A.v. Monoclonal antibody (JL-8), 632381) was from Clontech (Mountain View, CA). Anti-calsequestrin (PA1–913) was from Thermo Scientific. Anti-mouse IgG HRP (NA931V), anti-rabbit IgG HRP (HAF109, R&D Systems, Minneapolis, MI) were used as secondary antibody.

Fractionation—Rat hearts and cardiomyocyte cultures were fractionated according to the manufacturer (2145, Compartment protein extraction kit, Millipore).

Protein Extracts—Human and rat lysates were made in parallel to ensure identical treatment of the samples. Protein lysates from human biopsies were prepared using ice-cold PBS-based lysis buffer containing 1% Triton X-100 (X100-500ML, Sigma-Aldrich), 0.1% Tween 20 (161-0781, Bio-Rad), and protease and phosphatase inhibitors (Complete EDTA-free and Protease Inhibitor mixture tablets, Roche Applied Science). Biopsies were homogenized three times for 1 min on ice with a Polytron 1200 homogenizer and centrifuged at 100,000 g for 10 min at 4 °C. Supernatants were collected and stored at −70 °C. Frozen LVs from rats were pulverized in a mortar with liquid nitrogen before transfer to lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton) supplemented with 1 mM PMSF (93482, Sigma-Aldrich) and a Complete Mini EDTA-free tablet (Roche Applied Science). Protease inhibitors were omitted for rat LV lysate treated with active calpain. Tissue samples were homogenized three times for 1 min on ice with a Polytron 1200 homogenizer and centrifuged at 100,000 × g for 60 min at 4 °C. Supernatants were collected and stored at −70 °C. Protein concentrations were determined by the Micro BCA protein assay kit (Pierce).

DNA Constructs—Cloning and mutations of NCX1 and calpain-1 constructs were performed by Genscript. The Mammalian Gene Collection mouse clone BC079673 was used for NCX1 constructs. WT NCX1 (full-length) was cloned into pEGFP-N1 with and without a stop codon before GFP (Clontech) or into the first reading frame of pAdTrack-cytomegalovirus (CMV) shuttle vector (plasmid 16405, Addgene, Cambridge, MA). Alanine mutants (Ala¹²⁷→Ser and Ala³⁶⁴→Thr) were mutated into NCX1/pEGFP-N1. Deletion mutants of the cytoplasmic loop of NCX1 (NCX1(243–787), NCX1(243–705), NCX1(243–532), NCX1(243–460), and NCX1(243–402)) were cloned into pEGFP-C2 (Clontech). WT rat calpain-1 catalytic subunit (BC061880) was cloned into pCEP4 (Invitrogen), as were the C-terminal FLAG-tagged calpain-1(1–220), calpain-1(213–365), calpain-1(357–525), and calpain-1(519–713). The fidelity of the cloning procedure and mutagenesis were verified by sequence analysis (Genscript). The empty vectors pcDNA3.1 and EGFP-N1 were obtained from Invitrogen and Clontech, respectively. Tobacco etch virus (TEV) protease in pCS2MT was kindly provided by Prof. Pati (17).

HEK293 and Transient Transfection—HEK293 cells were cultured in DMEM (41965-039, Invitrogen) supplemented with 10% FBS (14-701F, BioWhittaker), 1% non-essential amino acids (10370-021, Invitrogen), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (penicillin/streptomycin, P4333, Sigma-Aldrich) and maintained in a 37 °C, 5% CO₂ humidified incubator. The HEK293 cells were transfected with DNA using Lipofectamine 2000 as instructed by the manufacturer (11668-019, Invitrogen). After 24 h, the cells were either subjected to cell lysis or transferred to coverslips for patch clamp experiments. Cell lysis buffer contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100 with complete protease inhibitor mixture tablets (Roche Applied Science). Protease inhibitors were omitted for HEK293 lysate treated with active calpain. The glass coverslips were precoated with poly-L-lysine (P4707, Sigma-Aldrich), and the transfected cells on the coverslips were incubated for 24 h prior analysis. GFP was used as a positive control for the transfection.

Peptide Synthesis—Peptides were synthesized to >80% purity by Genscript: anti-NCX1 blocking peptide (amino acids 655–672), CGQPVRKFVKHARDHPIZIPST; anti-Met₃⁶⁹-NCX1 blocking peptide (amino acids 366–379), TRLMTGAGNILKRH; Biotin-calpain-1 (amino acids 29–48), GRHENAIKYL, Biotin-calpain-1 (amino acids 277–296), TDAKQVTYQQGQRNVLRMNR; Biotin-calpain-1 (amino acids 425–444), QKHRRRFRFGDMDTGFA; Biotin-calpain-1 (amino acids 453–472), AGQPVHLKRDFFLANASRQA; Biotin-calpain-1 (amino acids 469–488), SRAQSEFHNRELVSNRILR; Biotin-calpain-2 (amino acids 267–286), TGAEEVSSGSLQKLIIR; Biotin-NCX1 (amino acids 251–270)-XIP region, RRLFYKYVYKRYRAGKQRG (18); Biotin-calpain-1 (amino acids 29–48), GRHENAIKYL, Biotin-calpain-1 (amino acids 277–296), TDAKQVTYQQGQRNVLRMNR; Biotin-calpain-1 (amino acids 425–444), QKHRRRFRFGDMDTGFA; Biotin-calpain-1 (amino acids 453–472), AGQPVHLKRDFFLANASRQA; Biotin-calpain-1 (amino acids 469–488), SRAQSEFHNRELVSNRILR; Biotin-calpain-2 (amino acids 267–286), TGAEEVSSGSLQKLIIR; Biotin-NCX1 (amino acids 251–270)-XIP region, RRLFYKYVYKRYRAGKQRG (18); Biotin-NCX1 (amino acids 354–373), QKQKSRFYRIQATRLMTGAG.

Spot Membrane Synthesis—The intracellular loop (amino acids 243–799) of rat NCX1 protein (EDM02743) and full-length rat calpain-1 (BC061880) were synthesized as 20-mer peptides with 3-amino acid offsets on cellulose membranes using a Multipep automated peptide synthesizer (INTAVIS Bioanalytical Instruments AG, Köln, Germany) as described (19).

Calpain and NCX1 Overlay—The peptide array membranes were blocked in 1% casein in TBST (Tris-buffered saline with 1% Tween) overnight at 4 °C. The calpain and NCX1 overlay was conducted by incubating the membranes with 1 μg/ml recombinant His-TF-NCX₁ cyt (in 1% casein) or 1 μg/ml recombinant calpain-1 (human erythrocytes, 208713, Calbiochem) in 1X calpain buffer: 10 mM EGTA, 0.1% Triton, 20 mM HEPES (pH 7.5), and 20 mM CaCl₂. Thereafter, the membranes were washed five times in TBST for 5 min before binding was detected by immunoblotting.

Pull-down Assay—Monoclonal anti-biotin-conjugated beads (A-1559, Sigma-Aldrich) were incubated with 8 μM peptides in...
100 μl of PBS for 2 h with rotation. The beads were then washed three times with PBS before incubation with 1 μg of recombinant calpain-1 (Calbiochem) for 2 h at 4 °C. The protein complexes were subsequently washed three times in lysis buffer and boiled for 5 min at 96 °C in 2× SDS loading buffer before SDS-PAGE analysis.

**Immunoprecipitation**—Immunoprecipitations were performed using 2 μg of the appropriate antibody. The immunocomplexes were collected by protein A/G-agarose beads (sc-2003, Santa Cruz Biotechnology), washed three times in immunoprecipitation buffer, and boiled in SDS loading buffer before SDS-PAGE analysis. Equal amounts of rabbit IgG (sc-2027) and mouse IgG (sc-2025, Santa Cruz Biotechnology) were used as negative controls.

**Proximity Ligation Assay and Image Acquisition**—Isolated adult rat cardiomyocytes were plated on laminin (Sigma-Aldrich)-coated glass coverslips and left to adhere for 1 h. The cells were fixed in 4% paraformaldehyde (158127, Sigma-Aldrich), permeabilized with 0.3% Triton X-100 (X100, Sigma-Aldrich), and incubated with anti-NCX1 (custom-made, GenScript) and anti-calpain-1 (ab98778, Abcam) overnight at 4 °C. Staining with a custom-made NCX1 blocking peptide (GenScript) or staining in the absence of primary antibodies served as a negative control. The proximity ligation assay (PLA) was performed using the Duolink kit according to the manufacturer’s protocol (Sigma-Aldrich). In brief, species-specific secondary antibodies, called PLA probes, were added to the cells. These PLA probes, each having a unique short DNA strand attached to it, bound to the primary antibodies. When the PLA probes were in close proximity, <40 nm, the DNA strands hybridized when enzyme ligase solution was added. The signal was amplified via rolling circle amplification using an amplification solution consisting of polymerase, nucleotides, and fluorescence-labeled oligonucleotides. The output signal was visible as distinct fluorescent green spots because we used the green Duolink®, In Situ detection reagent (excitation 488 nm, emission 510 nm). The cells were then incubated with 600-nm SYTOX Orange (S-11368; Molecular Probes, Inc., Eugene, OR) (excitation 543 nm, emission 650 nm), a nucleic acid stain, for 10 min at room temperature and rinsed three times for 5 min with PBS. Imaging experiments were performed at 25 °C. The water-based In Situ Mounting Medium (refractive index 1.44), provided in the kit was used to mount the glass coverslip to the glass slide. The cells were visualized with an inverted LSM 710 confocal microscope (Zeiss GmbH, Jena, Germany) equipped with a LD C-Apochromat ×40 objective (numerical aperture 1.1). Sequential optical scans were acquired using the Zeiss ZEN imaging software.

**Calpain-1 and AcTEV Cleavage in Vitro**—Rat heart extract or HEK293 lysates were diluted (1:1) in 2× calpain buffer (1× calpain buffer: 10 mM EGTA, 0.1% Triton, 20 mM HEPES (pH 7.5), and 20 mM CaCl₂). Subsequently, 1 μg of calpain-1 (Calbiochem) was added, and proteolysis was performed at 37 °C for 1 h. To inhibit calpain, lysates were incubated with 5 μM calpastatin (Millipore) at 37 °C for 30 min before adding active calpain. The reaction was stopped by adding 4× SDS loading buffer. Cleavage was analyzed by SDS-PAGE and immunoblotting. AcTEV cleavage was conducted according to the instructions provided by the manufacturer (12575-015, Invitrogen).

**Ca²⁺ Treatment of Cardiomyocytes**—To induce activation of endogenous calpain, 4 mM CaCl₂ was added to the neonatal cardiomyocyte culture. To inhibit calpain, 10 μM calpeptin (Calbiochem) was added prior to the addition of CaCl₂. After a 24-h incubation, the membrane proteins were isolated using the Compartment protein extraction kit (Millipore).

**Immunoblotting**—Lysates and immunoprecipitates were analyzed on 4–15 or 15% SDS-PAGE Criterion (Bio-Rad) and blotted onto PVDF membranes (RPN 303F, GE Healthcare). The PVDF membranes and peptide arrays were blocked in 5% nonfat dry milk or 1% casein in TBST for 60 min at room temperature, incubated overnight at 4 °C with primary antibodies, washed 3–5 times for 5 min in TBST, and incubated with an HRP-conjugated secondary antibody. Blots were developed by using ECL Plus or Prime (RPN 2132 or RPN 2232, GE Healthcare). The chemiluminescence signals were detected by Las 1000 or Las-4000 (Fujifilm, Tokyo, Japan).

**Bioinformatics**—The CaMPDB (Calpain for Modulatory Proteolysis Database) with PSSM and SVM (RBF Kernel) prediction models were used to identify putative calpain cleavage sites in the cytoplasmic region of NCX1 (20).

**Patch Clamp Experiments**—Whole cell patch clamp experiments were conducted with either pEGFP-N1- or pAdTrack-CMV-transfected HEK293 cells using an Axoclamp 2B amplifier (Axon Instruments) and low resistance pipettes (1–2.5 megaohms). The recordings were performed in an extracellular solution containing 140 mM NaCl, 5 mM CsCl, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 5 mM CaCl₂, 10 mM HEPES, 10 mM glucose, pH 7.4 (CsOH), and osmolality 290 mosM. K⁺, Ca²⁺, Cl⁻, and Na⁺-K⁺-ATPase currents were blocked by inclusion of cesium, nifedipine (20 μM), niflumic acid (30 μM), and ouabain (1 mM), respectively, in the solution. Patch pipettes were filled with a solution containing 100 mM cesium glutamate, 1 mM MgCl₂, 10 mM HEPES, 2.5 mM Na₂-ATP, 10 mM EGTA, CsCl₂, pH 7.2, and osmolality 270 mosM. The NCX1 reversal potential under these conditions was −43 mV at 37 °C. Cells were voltage-clamped at −43 mV for 4–5 min to allow sufficient intracellular dialysis. Following a prepulse protocol, NCX1 current was elicited by a descending voltage ramp from 120 to −100 mV. The NCX1 current was calculated as the difference current prior to and following the application of 5 mM Ni²⁺.

**Structural Modeling**—Atomic coordinates for rat calpain in complex with leupeptin (PDB code 1TL9 (21)) and human calpain in complex with a peptidomimetic inhibitor (PDB code 1ZCM (22)) were used to build models of the 3420TRLM369 and 366TRLM3770 binding to calpain. The peptide models were refined using the Rosetta FlexPepDock server (23). For the leupeptin-based model, the rat protein was replaced by human calpain before submitting the input structure to FlexPepDock. Values of the top scoring complexes calculated by FlexPepDock were analyzed and visualized by PyMOL (Schrödinger LLC, New York).

**Densitometric Analysis**—Densitometric analysis was performed using Image Gauge version 4.0, ImageQuant TL (Amer sham Biosciences), or ImageJ (National Institutes of Health).
Calpain Cleavage and Inactivation of NCX1 in HF

### RESULTS

**Increased Levels of NCX1 and a 75-kDa NCX1 Fragment after Chronic Pressure Overload**—NCX1 was analyzed in LV lysates isolated from patients with AS and rats 6 weeks after aortic banding by immunoblotting. Patient and animal clinical characteristics are listed in Tables 1 and 2, respectively. Full-length NCX1 and a proteolytic 75-kDa NCX1 fragment were increased 2.0- and 3.7-fold in AS patients with myocardial hypertrophy, respectively, compared with controls (Fig. 1C). Similarly, rats with hypertrophic, non-failing (ABHT) hearts showed a somewhat increased level of both full-length NCX1 and 75-kDa NCX1 fragment, whereas both levels were significantly increased in failing (ABHF) hearts compared with sham-operated controls (Fig. 1B). The increase in NCX1 fragmentation was paralleled by increased proteolysis of PKCα, a known substrate for calpain (24), implying increased calpain activity in ABHT and ABHF hearts. Calpain cleavage. No signal was observed when primary antibodies to NCX1 (13) and calpain antibodies (Fig. 3C), or anti-NCX1 was preincubated, and NCX1 levels in LV lysates (Figs. 1, A and B), NCX1 levels tended to be elevated in ABHT membrane fractions, and were significantly increased in ABHF (Fig. 2, B and C, top panels). Although calpain expression also was increased in membrane fractions before pressure overload (Fig. 2, B and C), immunoprecipitation showed that less calpain precipitated with NCX1 in ABHF compared with sham (Fig. 2D). Taken together, our data indicate that calpain associates with NCX1 in membrane fractions in normal and hypertrophic hearts but might dissociate after cleavage of NCX1 in HF.

### Association of Calpain-NCX1 in Cardiac Membranes—Although calpain is predominantly a cytoplasmic protease (25), it was also observed together with NCX1 in membrane fractions from rat LV (Fig. 2A, left, weak signal) and cardiomyocytes (right, strong signal). To test whether calpain was recruited to membrane fractions after chronic pressure overload, subcellular fractionation of LV from sham, ABHT, and ABHF hearts was performed. Immunoblotting showed that calpain levels were increased by 2.3- and 1.8-fold in ABHT and ABHF membrane fractions, respectively (Fig. 2B and 2C, middle). Consistent with findings in LV lysates (Figs. 1, A and B), NCX1 levels tended to be elevated in ABHT membrane fractions and were significantly increased in ABHF (Fig. 2, B and C, top panels). Although calpain expression also was increased in membrane fractions before pressure overload (Fig. 2, B and C), immunoprecipitation showed that less calpain precipitated with NCX1 in ABHF compared with sham (Fig. 2D). Taken together, our data indicate that calpain associates with NCX1 in membrane fractions in normal and hypertrophic hearts but might dissociate after cleavage of NCX1 in HF.

### Statistics—All data were expressed as mean ± S.E. Comparisons between two groups were analyzed using unpaired Student’s t test or Mann-Whitney test (GraphPad Software). A p value of <0.05 was considered statistically significant.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>AS</th>
<th>CAD</th>
</tr>
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<tbody>
<tr>
<td>No. of patients (no. of females)</td>
<td>8 (4)</td>
<td>8 (1)</td>
</tr>
<tr>
<td>Years of age at myocardial tissue biopsy</td>
<td>74.88 ± 2.53</td>
<td>68.34 ± 2.65</td>
</tr>
<tr>
<td>Ejection fraction</td>
<td>All &gt;50%</td>
<td>All &gt;50%</td>
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<tr>
<td>Aortic valve area (cm²)</td>
<td>0.66 ± 0.05</td>
<td>NA</td>
</tr>
<tr>
<td>Maximum aortic gradient (mm Hg)</td>
<td>85.38 ± 7.91</td>
<td>NA</td>
</tr>
<tr>
<td>Mean aortic gradient (mm Hg)</td>
<td>54.63 ± 4.50</td>
<td>NA</td>
</tr>
<tr>
<td>LVIDd (cm)</td>
<td>4.83 ± 0.25</td>
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</tr>
<tr>
<td>LVd (cm)</td>
<td>2.88 ± 0.29</td>
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</tr>
<tr>
<td>IVSd (cm)</td>
<td>1.16 ± 0.07</td>
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</tr>
<tr>
<td>LVPWd (cm)</td>
<td>1.09 ± 0.03</td>
<td>NA</td>
</tr>
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### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>ABHT</th>
<th>ABHF</th>
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<tbody>
<tr>
<td>Number (n)</td>
<td>14</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>357 ± 6</td>
<td>332 ± 7a</td>
<td>338 ± 13</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.96 ± 0.03</td>
<td>1.53 ± 0.05a</td>
<td>2.7 ± 0.13ab</td>
</tr>
<tr>
<td>LV weight (g)</td>
<td>0.56 ± 0.03</td>
<td>0.97 ± 0.02</td>
<td>1.3 ± 0.05ab</td>
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<tr>
<td>LV/body weight ratio (mg/g)</td>
<td>1.58 ± 0.08</td>
<td>2.99 ± 0.09</td>
<td>4.0 ± 0.22ab</td>
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<td>Lung weight (g)</td>
<td>1.11 ± 0.06</td>
<td>1.55 ± 0.04a</td>
<td>4.0 ± 0.14ab</td>
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<td>LVDD (mm)</td>
<td>6.2 ± 0.1</td>
<td>5.4 ± 0.1a</td>
<td>7.5 ± 0.2a</td>
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<tr>
<td>LVDS (mm)</td>
<td>3.0 ± 0.2</td>
<td>2.2 ± 0.1a</td>
<td>4.5 ± 0.3a</td>
</tr>
<tr>
<td>FS (%)</td>
<td>57 ± 2</td>
<td>59 ± 2</td>
<td>40 ± 3ab</td>
</tr>
<tr>
<td>PWd (mm)</td>
<td>1.6 ± 0.04</td>
<td>2.3 ± 0.1a</td>
<td>2.4 ± 0.1a</td>
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<tr>
<td>LAD (mm)</td>
<td>3.4 ± 0.1</td>
<td>4.2 ± 0.1a</td>
<td>6.1 ± 0.2a</td>
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<tr>
<td>Peak mitral flow (mm/s)</td>
<td>859 ± 50</td>
<td>925 ± 47</td>
<td>1076 ± 42ab</td>
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<tr>
<td>Mitral deceleration (mm/s)</td>
<td>2714 ± 145</td>
<td>3314 ± 242a</td>
<td>4940 ± 418a</td>
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<td>Peak LVOT flow (mm/s)</td>
<td>1087 ± 62</td>
<td>917 ± 39a</td>
<td>824 ± 90</td>
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<tr>
<td>Heart rate</td>
<td>430 ± 6</td>
<td>416 ± 12</td>
<td>383 ± 8</td>
</tr>
<tr>
<td>CO in LVOT (ml/min)</td>
<td>129 ± 6</td>
<td>115 ± 7</td>
<td>103 ± 12</td>
</tr>
<tr>
<td>Maximum velocity (mm/s)</td>
<td>73 ± 3</td>
<td>51 ± 3a</td>
<td>38 ± 4ab</td>
</tr>
<tr>
<td>Minimal velocity (mm/s)</td>
<td>78 ± 3</td>
<td>56 ± 4a</td>
<td>51 ± 3a</td>
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</table>

* p < 0.05 versus sham.
* p < 0.05 versus ABHT.
in Fig. 3E) together with recombinant His-TF-NCX1\textsubscript{cyt} were performed with anti-biotin-agarose beads. Only calpain(277–296) and calpain(425–444), residing in catalytic domain IIb and domain III, respectively, co-precipitated His-TF-NCX1\textsubscript{cyt} (Fig. 3F). Thus, the other sequences were revealed as false positives.

Interestingly, NCX1 bound to catalytic domain IIb, containing His\textsubscript{272} and Asn\textsubscript{296} (Fig. 3E, boldface type), which together with Cys\textsubscript{115} forms the catalytic triad in the active site in presence of Ca\textsuperscript{2+} (26). The NCX1-XIP region containing the self-dimerization domain (18) was used as a positive control for the assay. Beads without any peptide were used as a negative control.

The two NCX1-calpain interaction sites were also confirmed by binding studies in HEK293 cells. Five constructs were expressed in HEK293: calpain-1(FL)-FLAG, calpain-1(1–220)-FLAG, calpain-1(213–365)-FLAG, calpain-1(357–525)-FLAG, and calpain-1(519–731)-FLAG (Fig. 4A). Fishing with His-TF-NCX1\textsubscript{cyt} in HEK293 lysates containing each variant revealed that NCX1 bound specifically to calpain-1(213–365) and calpain-1(357–525), containing catalytic domain IIb and domain III, respectively (Fig. 4B, stars). NCX1 bound only very weakly to these calpain-1 domains compared with calpain-1(FL)-FLAG (top left), suggesting that both regions were required to obtain strong binding. No His-TF-NCX1\textsubscript{cyt} binding was observed to calpain-1(1–220)-FLAG or calpain-1(519–731)-FLAG, not even after longer time exposures.

Sequence alignments show that NCX1 binding site I in catalytic domain IIb (Fig. 4C) and site II in domain III (Fig. 4D) were highly conserved across human, rat, and mouse calpain-1. Although there was less sequence similarity between catalytic domain IIb of calpain-1 and calpain-2 (Fig. 4E), His-TF-NCX1\textsubscript{cyt} also bound calpain-2 (amino acids 267–286) (Fig. 4F), suggesting NCX1 to be both a calpain-1 and calpain-2 sub-
strate. Indeed, the substrate specificities have been shown to be almost indistinguishable (12).

Identification of a Novel Calpain Binding and Cleavage Site at Met369 in NCX1—By using the CaMPDB prediction database (20), two high score calpain cleavage sites were identified at Lys331 and Met369 in the catenin-like domain (CLD) of NCX1 (Fig. 5A). Cleavage of either site could theoretically result in an NCX1 fragment of \( \frac{1}{75} \) kDa. The two sites were each mutated to 10 alanines in NCX1-GFP (Fig. 5A, underlined) because this mutation strategy has been shown to inhibit calpain cleavage (24). NCX1(Ala327–336)-GFP (Fig. 5B), but not NCX1(Ala364–373)-GFP (Fig. 5C) was cleaved by calpain, suggesting Met369 to be a novel calpain cleavage site. The apparent higher molecular mass of cleaved fragment (95 kDa) was due to GFP tag. Moreover, NCX1 (Ala364–373)-GFP precipitated less calpain than NCX1-GFP (Fig. 5D), suggesting that an intact calpain cleavage site was required to obtain a strong calpain-NCX1 interaction. In an in vitro calpain cleavage assay, the presence of a substrate competitor peptide, NCX1(354–373), containing Met 369, reduced cleavage of NCX1-GFP (Fig. 5E). Finally, to confirm Met369 as the cleavage site, an antibody against the Met369 cleavage site was generated. Anti-Met 369-NCX1 detected NCX1 only when epitope TRLMTGAGNILKRH was intact (Fig. 5F). In contrast to the regular anti-NCX1, which detects both FL and 75-kDa NCX1 (Figs. 1–4 and 5G, top), anti-Met369-NCX1 detected only FL NCX1 (Fig. 5G, middle), suggesting that the 75-kDa fragment originates from cleavage at Met369. Preincubation of anti-Met369-NCX1 with a blocking peptide gave no signals from FL NCX1 or 75-kDa NCX1 (Fig. 5G, bottom).

Conclusively, our data indicate a novel calpain cleavage site at Met369 in NCX1-CLD. The sequence spanning Met369 is

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**FIGURE 2.** NCX1 and calpain levels in isolated cardiac membrane fractions. A, NCX1 and calpain were analyzed in cytoplasmic and membrane fractions isolated from rat LV and neonatal cardiomyocytes using anti-NCX1 and anti-calpain. GAPDH and EGFR were used as controls for cytoplasmic and membrane fractions, respectively. NCX1 and calpain were analyzed in membrane fractions isolated from ABHT (B) and ABHF (C) versus sham using anti-NCX1 and anti-calpain. D, lysates from sham, ABHT, and ABHF were subjected to immunoprecipitation (IP) using anti-NCX1. The presence of endogenous NCX1 and calpain in immunoprecipitates was analyzed by immunoblotting. B–D, differences were tested using unpaired Student’s t test (*, \( p < 0.05 \); **, \( p < 0.001 \)) (\( n = 4–6 \)). Error bars, S.E.
identical in human, rat, and mouse NCX1 (Fig. 5H) and highly conserved in NCX1–3 isoforms (Fig. 5I).

Cleavage at Met369 Reduces NCX1 Activity—To investigate the biological role of specific cleavage of NCX1 at Met369, we engineered cleavage without calpain activation. This was accomplished by replacing amino acids surrounding the calpain cleavage site with a TEV protease site (Fig. 6A), which cleaves amino acid sequence ENLYFQ(G/S) after Q with high specificity (27). Transient expression of NCX1(TEV), NCX1(Ala364–373), or NCX1(WT) into HEK293 cells. In contrast to NCX1(WT) (without GFP), which was cleaved to a fragment of 75 kDa, neither NCX1(TEV) nor NCX1(Ala364–373) was cleaved by active recombinant calpain-1 (data not shown). However, transient expression of NCX1(TEV) with TEV protease into HEK293 resulted in generation of the 75-kDa proteolytic NCX1 fragment in membrane fraction (Fig. 6B, denoted with stars).

Total NCX1 current before and after TEV protease cleavage was analyzed by using whole cell patch clamp techniques. NCX1 and NCX1(TEV) in combination with TEV protease were transiently expressed into HEK293. NCX1 current was isolated by

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FIGURE 3. NCX1 binding in calpain. A, confocal images of in situ proximity ligation assay. NCX1-calpain-1 association analyzed in adult cardiomyocytes using anti-NCX1 and anti-calpain (left; interaction indicated by green spots; see “Experimental Procedures” for details). Incubation without primary antibodies (middle) and preincubating anti-NCX1 with blocking peptide (right) were used as negative controls. Nuclei were stained with SYTOX orange. B, epitope was identified by overlaying an array of immobilized overlapping 20-mer calpain catalytic subunit peptides with anti-calpain (sc-30064, top). Amino acids 201–232 are relevant for anti-calpain binding and are within the 230-amino acid fragment used as antigen by the manufacturer (Santa Cruz Biotechnology, Inc.). Immunoblotting with anti-calpain was used as a negative control (bottom). C, membrane fraction was subjected to immunoprecipitation using anti-calpain or control rabbit IgG (n = 3). Lysates and immunoprecipitates were analyzed for the presence of endogenous NCX1 and calpain by immunoblotting. D, schematic illustration of recombinant His-TF-NCX1cyt (top). Shown is immunoprecipitation (IP) of recombinant calpain-1 with His-TF-NCX1cyt using anti-His (bottom). Lysates and precipitates were analyzed for the presence of calpain and NCX1 by immunoblotting. E, NCX1 binding identified by overlaying His-TF-NCX1cyt on membranes containing 20-mer overlapping calpain-1 peptides and immunoblotting with anti-NCX1 (n = 3). Incubation without His-TF-NCX1cyt was used as a negative control (bottom). Underlined amino acids indicate sequences used in the pull-down experiment in F. F, pull-down assays with biotinylated calpain peptides against His-TF-NCX1cyt were performed using anti-NCX1. Incubation without His-TF-NCX1cyt was used as a negative control (bottom).
measuring Ni²⁺-sensitive current elicited during a slow voltage ramp from 120 to −100 mV (Fig. 6C). Plotted current-voltage relationships show reduced current in both forward and reverse mode in cleaved NCX1(TEV) (Fig. 6D). Non-cleaved NCX1(TEV) showed the same current profile as NCX1(WT) (data not shown). Taken together, our data strongly suggest that cleavage of NCX1 at Met³⁶⁹ inhibits NCX1 activity.

Structural Modeling of the NCX1-Met³⁶⁹ Peptide into the Calpain Active Cleft—Upon Ca²⁺ binding, calpain domains Ila and IIb move toward each other to form an active narrow cleft that substrate fits into (28). Peptide docking models of 366TRLM³⁶⁹ or 366TRLMT³⁷⁰ peptides from NCX1 onto human calpain are shown in Fig. 7. Docking of 366TRLMT³⁷⁰, using leupeptin as a template (21), showed that Met³⁶⁹ fitted well in the cleft between domain Ila and IIb in calpain (Fig. 7A). In this model, the scissile peptide bond was not positioned close to catalytic Cys¹¹⁵. However, docking the shorter 366TRLM³⁶⁹ peptide, using a peptidomimetics inhibitor as a template (22), gave a model where the peptide bond was positioned close to Cys¹¹⁵ (Fig. 7B). Alignment of the NCX1 binding site in rat calpain-1 versus calpain-2 catalytic domain. His²⁷² and Asn²⁹⁶ in the catalytic cleft are indicated with arrows. Black boxes, functional similar amino acids in C-E (DNA Star, Madison, WI).

In this model, the scissile peptide bond was not positioned close to catalytic Cys¹¹⁵. However, docking the shorter 366TRLM³⁶⁹ peptide, using a peptidomimetics inhibitor as a template (22), gave a model where the peptide bond was positioned close to Cys¹¹⁵ (Fig. 7B). In this latter model, the NCX1-Met³⁶⁹ residue was still observed to bind in the cleft but was not positioned as close to surface of calpain. This model, which was based on a covalently linked inhibitor, did not give any indication as to where the C-terminal part of the NCX1 peptide would be located. Inspection of several top ranked docking models suggested two alternative positions for the Arg³⁶⁷ residue (Fig. 7B). Both peptide docking models have the Leu³⁶⁸ residue positioned in the same place as the corresponding Leu residues in leupeptin and peptidomimetics inhibitor templates (21, 22).
Identification of a Second Calpain Binding Site in the NCX1-CBD1—As indicated in Figs. 3 and 4, calpain domain III also bound to NCX1. To identify domain III binding in NCX1, NCX1-GFP and GFP-NCX1 variants were designed and expressed in HEK293: NCX1(FL)-GFP, GFP-NCX1(243–787), GFP-NCX1(243–705), GFP-NCX1(243–532), and GFP-NCX1(243–402) (Fig. 8A). Fishing with active calpain-1 revealed that all GFP-NCX1 variants precipitated, except GFP-NCX1(243–402) (Fig. 8B). This finding suggested the second calpain binding site to be within amino acids 402–460 in NCX1. This observation was confirmed by overlaying calpain-1 onto NCX1cyt spot-synthesized as 20-mer overlapping peptides on membranes. Immunoblotting with anti-calpain identified calpain binding to amino acids 345–373 and 411–439 in NCX1 (Fig. 8C). Importantly, amino acids 345–373 contain the novel calpain binding and cleavage site, Met369, identified above. The second calpain binding site resided within the first regulatory calcium binding region of NCX1(CBD1).
Sequence alignments show that the second calpain binding site was almost identical in human, rat, and mouse NCX1 (Fig. 8D) and was similar between rat NCX1–3 isoforms (Fig. 8E). A model of calpain binding and cleavage of NCX1 is summarized in Fig. 9.

**DISCUSSION**

In an ongoing effort to understand regulation of cardiac NCX1 activity, we sought to identify molecular mechanisms and consequences of calpain binding and cleavage of NCX1 in normal, hypertrophic non-failing, and failing hearts.

**Calpain Activation and Cleavage of Cardiac NCX1**—We observed increasing levels of a 75-kDa proteolytic NCX1 fragment along with FL NCX1 in LV lysates isolated from aortic-banded rats with hypertrophic, non-failing heart (ABHT) and HF (ABHF). Similar results were observed in LV biopsies from aortic stenosis (AS) patients with hypertrophic remodeling. The observed increase in NCX1 fragmentation was paralleled by an increase in calpain levels, an observation consistent with previous reports on involvement of calpains in various heart diseases (29). Moreover, calpain interacted directly with NCX1 in membrane fractions, but the interaction was reduced in ABHF compared with sham, suggesting that calpain dissociated from NCX1 after cleavage. A low level of the 75-kDa proteolytic NCX1 fragment was also observed in sham-operated rats, consistent with a low level of proteolytic calpain activity in non-stressed myocardium (30).

The precise activation mechanism of calpains is not known. Calpain is believed to predominately present as an inactive enzyme in the cytosol, where it binds to the endogenous inhibitor calpastatin. When Ca\(^{2+}\) rises, calpain translocates to membrane, where it associates with phospholipids. Calpain-1 activation reportedly requires 3–50 \(\mu M\) \([Ca^{2+}]_i\), whereas calpain-2 requires 400–800 \(\mu M\) \([Ca^{2+}]_i\) (8). Because bulk cytosolic \([Ca^{2+}]_i\) is only \(\sim 0.1 \mu M\) in resting cells, it is believed that calpain activation occurs at epicenters of \([Ca^{2+}]_i\) influx and/or release and then quickly inactivates as \([Ca^{2+}]_i\) diffuses away or is removed by various pumps (8). In cardiomyocytes, NCX1 localizes to surface sarcolemma and T-tubules at dyadic junctions with the sarcoplasmic reticulum (SR). Opening of L-type \(Ca^{2+}\) channels during action potential triggers a large release of \(Ca^{2+}\) from SR via clusters of ryanodine receptors, which has been estimated to result in peak dyadic \([Ca^{2+}]_i\) concentrations between 10 and 15 \(\mu M\) (31). Thus, systolic dyadic \([Ca^{2+}]_i\) appears to be sufficient for at least calpain-1 activation and cleavage of neighboring NCX1. However, \textit{in situ} experiments indicate that increasing the mean cytosolic \([Ca^{2+}]_i\) to as little as 451 nM \(Ca^{2+}\) can activate calpain in cardiac myocytes (32). Thus, there may be additional activators of calpain present in...
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A Novel Inhibitory Calpain Cleavage Site at NCX1-Met\(^{369}\) — Our screening strategy utilizing peptide arrays combined with bioinformatics is an effective approach to identify novel binding and cleavage sites in proteins. Calpain-1 catalytic domain was observed to bind to amino acids 345–373 in the disordered CLD, resulting in cleavage at NCX1-Met\(^{369}\). In support of our assertion that NCX1-Met\(^{369}\) is a novel calpain cleavage site, we observed that 1) CaMPDB prediction database (20) predicted Met\(^{369}\) to be a high score cleavage site; 2) cleavage of Met\(^{369}\) resulted in a 75-kDa NCX1 fragment, which is of a size similar to that reported for proteolytic NCX1 digestion in LV lysate (13), brain (9), isolated caveolae vesicles, and endoplasmic reticulum (ER) from bovine pulmonary artery smooth muscle (14, 33); 3) calpain bound directly to the NCX1-Met\(^{369}\)-containing region using peptide arrays; 4) mutation of the region spanning Met\(^{369}\) into alanines or TEV protease cleavage site inhibited calpain cleavage of NCX1, thus suggesting that Met\(^{369}\) is the sole or primary cleavage site; 5) an excess of a soluble substrate peptide containing the region spanning Met\(^{369}\) blocked cleavage of NCX1; and 6) antibody with an epitope covering the Met\(^{369}\)-spanning sequence was not able to detect the endogenous 75-kDa NCX1 fragment.

Engineering Met\(^{369}\) into a TEV recognition sequence enabled us to explore the biological effect of NCX1-Met\(^{369}\) cleavage without calpain activation. Cleavage of NCX1-Met\(^{369}\) led to a reduction in NCX1 activity (both forward and reverse mode) compared with full-length NCX1 when expressed in HEK293. Contrary to our findings, calpain treatment was reported to lead to constitutive activation of cardiac NCX1 when expressed in Xenopus oocytes (34). However, the authors reported that the results could not be replicated when Ca\(^{2+}\)-binding sites in CBD1 or CBD2 were mutated. Thus, in light of these data, we suggest that calpain is able to activate full-length NCX1 through an indirect mechanism. For example, PKCa, which phosphorylates and activates NCX1 (35), is reported to be constitutively activated by calpain cleavage, leading to hyperphosphorylation of its substrates (24).

Calpain Cleavage of Other Ion Channels — Other Ca\(^{2+}\) transporters are also suggested to be substrates for calpain cleavage. These include the plasma membrane Ca\(^{2+}\)-ATPase, which, like NCX1, extrudes Ca\(^{2+}\) (36, 37), SR Ca\(^{2+}\)-handling proteins like RYR2 (11, 38) and SERCA2 (11, 39), mitochondrial inner membrane NCLX (10), ER NCX1 (33), and brain NCX3 (9). However, there is, in general, a paucity of data concerning the precise mechanisms and sites at which calpain cleaves these proteins. Functional consequences are also poorly understood, although it is generally suggested that the activity of Ca\(^{2+}\) transporters is inhibited following cleavage. Plasma membrane Ca\(^{2+}\)-ATPase has been shown to be sequentially cleaved, resulting in an initial functional activation as the C-terminal autoinhibitory domain was cleaved (36), followed by a secondary cleavage resulting in functional inhibition (40). Moreover, although cleavage of NCLX (110 kDa) and ER NCX1 led to Ca\(^{2+}\) overload in mitochondria (10) and inhibited Ca\(^{2+}\) uptake into ER (33), no mechanisms or cleavage sites were identified. Cleavage of NCX3 after brain ischemia contributed to glutamate-mediated neuronal cytotoxicity (9). A cluster of three calpain cleavage sites, within a region without any homology to NCX1, was reported to lead to constitutive activation of cardiac NCX1 isoforms in vivo (9). Consistent with their observations, no calpain binding was observed at NCX1-Lys\(^{377}\) in our study using peptide arrays.

Calpain Domain III Binds to the First Ca\(^{2+}\) Binding Region in NCX1(CBD1) — We identified that calpain domain III bound to amino acids 411–439 within NCX1-CBD1. Domain III, which is also referred to as the C2-like domain, contains a \(\beta\)-sandwich tertiary structure, similar to although with a different topology compared with known C2 domains found in enzymes that transiently bind to the membrane. Domain III is suggested to be involved in membrane translocation of calpain (41). However, to our knowledge, NCX1 is the first protein described to bind to domain III, suggesting an anchoring role of this domain in calpain.

NCX1-CBD1 is known to be a high affinity Ca\(^{2+}\) binding site and constitutes a crucial site for Ca\(^{2+}\) sensing leading to NCX1 activation. CBD1-Glu\(^{385}\), residing in the domain III binding
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site, has been shown to be important for coordinating Ca\(^{2+}\) binding (42). Thus, calpain anchoring might impair Ca\(^{2+}\) binding and consequently NCX1 function. It has been recently suggested that Ca\(^{2+}\) binding to CBD1 constrains the conformational freedom of CBD1-CBD2, rigidifying the intracellular loop and thus propagating the signal to ion transport sites in membrane (5). Because the calpain binding site in CBD1 was less conserved in NCX2 and NCX3, the presented calpain binding and cleavage mechanisms might be unique to NCX1.

Is Calpain Cleavage of Cardiac NCX1 a Compensatory Mechanism?—Inhibition of NCX1 might be beneficial in pathophysiological conditions or etiologies where increased NCX1 activity contributes to cardiac dysfunction. NCX and SERCA compete for the same pool of Ca\(^{2+}\), and increased NCX1 activity during conditions such as HF is believed to attenuate SR Ca\(^{2+}\) load and reduce the magnitude of Ca\(^{2+}\) transients (43). Indeed, NCX1 blockade has been observed to augment SR content and Ca\(^{2+}\) transients (44), and similar compensatory effects might be expected to result following calpain cleavage of NCX1. However, the specific phenotype of HF should be considered because there has been a recent appreciation that more than 50% of HF patients exhibit normal systolic function but impaired diastolic function (45). Reduced NCX1 activity can promote slowed removal of cytosolic Ca\(^{2+}\), leading to slowed cardiomyocyte relaxation during HF, and this deficit may become particularly significant when SERCA levels are also reduced (46). Although it is as yet unclear whether calpain-mediated cleavage may promote such dysfunction, reduced NCX1 activity is also known to result from elevated intracellular [Na\(^+\)] in failing cells due to reduced expression of the Na\(^+\)-K\(^+\) ATPase (47), augmentation of late Na\(^+\) current (48), and intracellular acidosis (49). Thus, there is general agreement that reduced NCX1 activity, such as we have observed following cal-

FIGURE 8. Calpain binding in NCX1. A, schematic illustrations of NCX1 variants expressed in HEK293 cells. Shown are the CLD as well as CBD1 and CBD2 (7, 42). B, immunoprecipitation (IP) of calpain-1 with NCX1 variants using anti-calpain. Input lysates and precipitates were analyzed with immunoblotting. C, identification of calpain binding by overlaying calpain-1 on 20-mer overlapping NCX1 peptides synthesized on membrane (n = 3). Binding was analyzed using anti-calpain. Incubation without calpain was used as a negative control (bottom). Shown is an alignment of calpain binding site II in human, rat, and mouse NCX1 (D) and rat NCX1–3 (E). Black boxes, functionally similar amino acids (DNA Star).
pain-induced NCX1 cleavage, is expected to be compensatory for systolic function in HF but detrimental for diastolic function.

Inhibition of NCX1 activity may also have potential beneficial effects in the setting of arrhythmia. Spontaneous SR Ca\(^{2+}\) release occurs more frequently during conditions such as HF, and resulting extrusion of released Ca\(^{2+}\) by NCX1 results in delayed afterdepolarizations. Reducing NCX1 activity can reduce the magnitude of these depolarizing currents, which will reduce the likelihood that they will trigger action potentials and ectopic beats (50). Reducing NCX1 current also shortens action potential, which makes the occurrence of early after-depolarizations less likely (50). Finally, inhibition of NCX1 current during ischemia and reperfusion may also be compensatory, where NCX1-mediated Ca\(^{2+}\) entry is a key contributor to cellular damage.

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Increased NCX1 activity by a Specific PLM Binding Peptide

Development of a High Affinity Phospholemman Binding Peptide that Reverses Phosphorylated Serine 68 Phospholemman Inhibition of the Cardiac Sodium-Calcium Exchanger 1*

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*Running title: Increased NCX1 activity by a Specific PLM Binding Peptide

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Background: Interaction of phospholemman (PLM) with sodium-calcium exchanger 1 (NCX1) and its inhibition of NCX1 activity when phosphorylated at serine 68 (pSer68-PLM) is debated.

Results: Development of a high affinity PLM binding sequence that reverses pSer68-PLM inhibition of cardiac NCX1 in HEK293.

Conclusion: pSer68-PLM inhibits NCX1 activity directly.

Significance: The data suggest that pSer68-PLM is an NCX1 inhibitor in heart.
ABSTRACT

The cardiac sodium (Na\(^+\)) – calcium (Ca\(^{2+}\)) exchanger (NCX1) is an important regulator of intracellular Ca\(^{2+}\) and a potential therapeutic target in heart failure (HF). Phospholemman (PLM) is one of potential interacting partners regulating NCX1 activity. It is a transmembrane protein and a substrate for protein kinase A and protein kinase C. Several reports have demonstrated that binding of phosphorylated PLM (pSer68-PLM) to NCX1 leads to NCX1 inhibition, while other studies have failed to demonstrate a functional interaction of these proteins. In the present study, we aimed to analyze the biological function of pSer68-PLM-NCX1 interaction by developing high affinity blocking peptides. First, PLM was shown to co-fractionate and co-immunoprecipitate with NCX1 in rat left ventricle (LV) and in co-transfected HEK293 cells. PLM binding sites were consistently mapped to the previous reported NCX1-PASKT and NCX1-QKHPD regions by peptide array assays. Conversely, the two NCX1 regions bound to identical sequences in PLM cytoplasmic part, suggesting that NCX1-PASKT and NCX1-QKHPD bind to each PLM monomer. Using two-dimensional peptide arrays of the native NCX1 peptide sequence; 1-KHPDKEIEQLIELANYQVLS-20, revealed that single substitutions at position 1-11 (preferably with tryptophan or tyrosine) strongly increased pSer68-PLM binding affinity. Double substitution at position 1 and 4 with tyrosine (K1Y and D4Y) enhanced the pSer68-PLM affinity 8-fold. The optimized peptide blocked the binding of NCX1-PASKT and NCX1-QKHPD to PLM and reversed PLM (S68D) inhibition of NCX1 activity (both forward and reverse mode) in HEK293 cells. Altogether our data strongly suggest that PLM interacts directly with NCX1 and inhibits NCX1 activity when phosphorylated at serine 68.
INTRODUCTION

The cardiac Na\(^+\) Ca\(^{2+}\) exchanger (NCX1) is an important regulator for Ca\(^{2+}\) homeostasis and contractility. NCX1 is an ion transporter that has a bidirectional mode of operation. The primary operation mode of NCX1 is to extrude one Ca\(^{2+}\) in exchange for three Na\(^+\) ions (forward mode). In some conditions such as an increase in intracellular Na\(^+\) concentration and concomitant depolarization, reverse mode of NCX1 is favored (Ca\(^{2+}\) influx coupled to Na\(^+\) efflux) (1,2). The mode of NCX1 is tightly controlled by Ca\(^{2+}\) and Na\(^+\) ion concentrations. The contribution of NCX1 to Ca\(^{2+}\) removal is almost 30% in human and it varies between species (3). NCX1 expression and activity is altered in many models of cardiac hypertrophy and heart failure (HF), and the alterations depend on the species and the studied models of HF (4-7). NCX1 is a ~970 amino acids protein comprising of nine transmembrane segments (TMs) and a large cytoplasmic loop between TM5 and TM6. The cytoplasmic loop of NCX1 contains several regulatory domains including the inhibitory XIP region which binds to calmodulin (8), two calcium binding domains (CBD1 and CBD2) (9), potential sites for phosphorylation by PKC, Ca\(^{2+}\)/calmodulin-dependent kinase, and tyrosine kinase (10) and interaction sites for calpain (4) and phospholemman (PLM) (11).

Phospholemman (PLM) is a 72-amino acid phospho-protein belonging to the FXYD1 family of ion transport regulators (12) and it is the first reported endogenous inhibitor of NCX1 (13). PLM is highly expressed in heart (14), and it associates with NCX1 in cardiac membranes (15). PLM inhibits NCX1 activity when it is co-expressed with NCX1 (13,16). The cytoplasmic part of PLM interacts with PASKT- and QKHPD- containing sequences in the intracellular region of NCX1 (17) and it inhibits NCX1 when it is phosphorylated at serine 68 (pSer68-PLM) (18) by protein kinase A (PKA) or protein kinase C (PKC). In addition, PLM regulates NKA activity (as a non-phosphorylated monomer) (19) and potentially modulates L-type Ca\(^{2+}\) channels (20). These results imply that the interpretations of the effects of PLM and phosphorylated PLM on cardiac contractility are complex. PLM level is increased after myocardial infarction (MI) in rats (21) and a decrease in activity of NKA as well as NCX1 has been shown in MI rats (22-24). PLM phosphorylation level is also increased in rat hearts subjected to acute ischemia (25) and in an arrhythmogenic rabbit model of HF (26). Constitutive overexpression of phosphorylated PLM (S68E, mimic phosphorylated serine 68) in mice, results in arrhythmias, early mortality and HF (27). Since PLM regulates important ion transporters in the heart, it is considered as a novel drug target.

Although there is several strong evidence that pSer68-PLM is an endogenous NCX1 inhibitor (28), absence of interaction between PLM and NCX1 has been reported (29). In this present study, we aimed to investigate the functional interaction of pSer68-PLM and NCX1 by mapping the reciprocal binding sites and developing high affinity blocking peptides specific for the pSer68-PLM-NCX1 interaction. Our data demonstrated that the optimized peptide derived from NCX1 had an enhanced affinity for pSer68-PLM and reversed inhibition of NCX1 by PLM (S68D) in HEK293 cells.
**METHODS**

**Plasmid DNA** — The MGC mouse clone BC079673 (NCX1) was cloned into the first reading frame of pAdTrack-cytomegalovirus (CMV) shuttle vector (plasmid 16405, Addgene, Cambridge, MA, USA). Mouse phospholemman (PLM) (AF089734) and PLM mutated at serine 68 to an aspartic acid (S68D) were cloned with a C-terminal stop codon into pcDNA3.1/myc-HisA (Invitrogen, Carlsbad, CA) by Genscript (Corporation, Piscataway, NJ). The fidelity of the cloning and mutation procedures was verified by sequence analysis (Genscript).

**Peptide synthesis** — Peptides on cellulose membranes were synthesized using Multipep automated peptide synthesizer (INTAVIS Bioanalytical Instruments AG, Koeln, Germany) (30,31). Parts of the intracellular loop of rat NCX1 (EDM02743) and mouse PLM (Q9Z239) were synthesized as overlapping 20-mer peptides with three amino acid offsets. Peptides in solution were synthesized and purified to obtain > 80% purity by Genscript (Corporation, Piscataway, NJ). For some of the peptides a biotin tag was included at the N-terminus.

NCX1(261-280): KRYRAGKQRGMIEHEGDRP  
NCX1(267-286): KQRGMIEHEGDPRPASKTEI  
NCX1(324-343): ARILKELKQHPDKEIEQLI  
NCX1(333-352): KHPDKIEQIELANYQVLS  
PLMcycl (amino acids 58-92): KRCRCKFNQQRTGEPDEEEGTFRSSIRRLSSRRR  
pSer68-PLMcycl: KRCRCKFNQQRTGEPDEEEGTFRSSIRLPSSRRR  
NCX1(K333Y,D336Y): YHPYKEIEQIELANYQVLS  
NCX1(333-352) scrambled: VYEKNKLDIIAIEEQFISHI  
NCX1 blocking peptide: CGQPVFRKIVHARDHIPST

**Transfection of HEK293 cells** — HEK293 cells were cultured in DMEM (Gibco-BRL) supplemented with 10% fetal calf serum (Gibco-BRL), 100 unit/ml of penicillin, 0.1 mg/ml of streptomycin (penicillin/streptomycin, Sigma-Aldrich), 1% non-essential amino acids (Gibco-BRL) and maintained in a 37°C, 5% CO2 humidified incubator. Plasmid DNA was transfected into HEK293 cells using Lipofectamine 2000 as instructed by the manufacturer (Invitrogen) or CaCl2 method. After 24 hours, the cells were lysed in lysis buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA and 0.5% Triton X-100 with complete protease inhibitor cocktail tablets (Complete Mini EDTA-free, Roche Diagnostics, Mannheim, Germany). For patch clamp experiments, the 24h-transfected cells were transferred to pre-coated coverslips (pre-coated with poly-L-Lysine (P4707, Sigma-Aldrich)) and incubated for additional 24 hours in a 37°C, 5% CO2 humidified incubator.

**Preparation of rat left ventricle (LV) lysate** — Frozen LV from rats were pulverized in a mortar with liquid nitrogen followed by adding ice-cold lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 % Triton) supplemented with 1 mM PMSF (93482, Sigma-Aldrich) and complete protease inhibitor cocktail tablets (Complete Mini EDTA-free, Roche Diagnostics, Mannheim, Germany). Tissue samples were homogenized three times for 1 min on ice with a Polytron 1200 and centrifuged at 100 000 g for 60 min at 4°C. Supernatants were collected and stored at -70°C.

**Fractionation** — Rat LV and cardiomyocytes were fractionated according to manufacturer (2145, Compartment protein extraction kit, Millipore, MA).

**Overlay assay** — Synthesized peptide membranes were first activated by soaking membranes in methanol for few seconds and were furthermore washed three times with TBS-T (Tris-buffered saline with 0.1% tween). The membranes were then incubated with blocking solution (1% casein) (Roche Diagnostics, Mannheim, Germany) at room temperature. After one hour blocking, the membranes were incubated with 1-5 μM biotinylated peptide in 1% casein overnight at 4°C with gentle agitation. For the competition experiments, the blocking peptide (5 μM of NCX1(K333Y, D336Y)) was pre-incubated with the membranes overnight at 4°C with gentle agitation, before incubation with biotinylated peptide for 2 hours. The membranes were then washed three times with TBS-T (3×10min). Binding was detected by immunoblotting.
Increased NCX1 activity by a Specific PLM Binding Peptide

Pull-down assay with biotinylated peptides—Eight μM of each biotinylated peptide was incubated with 25 μl monoclonal anti-biotin conjugated beads (A-1559, Sigma-Aldrich, St. Louis, MO) in 100 μl 1x PBS for 2 hours at 4°C with rotation. To remove unbound peptide, the beads were washed three times with PBS, followed by adding 100 μl of HEK293 cell lysates, 0.5 μg of recombinant His-TF-NCX1 or 133 μM of PLM peptide diluted in 150 μl IP buffer containing 1% BSA. The samples were rotated for 2 hours at 4°C followed by washing the beads three times with IP buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100) prior to boiling in 2 x SDS loading buffer. Binding was analysed by immunoblottting.

Immunoprecipitation—Immunoprecipitation was performed by incubating 2 μg of the appropriate antibody with protein samples (rat heart lysates or HEK293 lysates) and protein A/G PLUS-Agarose (sc-2003, Santa Cruz Biotechnology) overnight at 4°C. Next day, the samples were then washed three times in IP buffer and boiled in 2 x SDS loading buffer before SDS-PAGE analysis. Equal amount of rabbit IgG (sc-2027) was used as negative control. Blocking peptide (antigen: cardiac NCX1, sequence: CGQPVFKVRHARDHIPST) (Genescript) was incubated with anti-NCX1 prior to immunoprecipitation (negative control).

Immunoblotting—Samples from pull-down assays were analyzed on 4-15% or 15% SDS-PAGE Criterion Gel (BioRad Laboratories, Inc., Hercules, CA) and blotted onto PVDF membranes (RPN 303F, GE Healthcare). The PVDF membranes and peptide arrays were blocked in 5% non-fat dry milk or 1% casein in TBS-T for 60 minutes at room temperature, and incubated overnight with primary antibody at 4°C. After incubation with primary antibody, the membranes were washed five times 5 minutes in TBS-T and further incubated with a horseradish-peroxidase-conjugated secondary antibody. The membranes were developed by using ECL Plus (RPN2132, GE HealthCare). The chemiluminescence signals were detected by LAS 1000 or LAS 4000 (Fujifilm, Tokyo, Japan).

Antibodies and recombinant protein—Anti-NCX1 (epitope: GQPVFRKVRHARDHIPST) was custom made from Genscript (Corporation, Piscataway, NJ). Anti-biotin-HRP (A0185) was purchased from Sigma-Aldrich (St. Louis, MO). Anti-PLM (anti-FXYD1) (ab76597) was purchased from Abcam (Cambridge, MA). Anti-GAPDH (V-18) (sc-20357) was from Santa Cruz Biotechnology, USA. Anticalsequestrin (PA1-913) was from Thermo Scientific. Anti-rabbit IgG HRP (NA934V, GE HealthCare) affinity purified polyclonal antibody and anti-goat IgG HRP (HA9109, R&D Systems, Minneapolis, MI) were used as secondary antibody. Recombinant cytoplasmic NCX1 protein with an N-terminal HIS tag and Trigger Factor (TF) to stabilize the protein (HIS-TF-NCX1) was custom made from Genscript Corp.

Patch-clamp experiments—Whole cell Patch-clamp experiments were conducted on NCX1 and PLM (S68D) transfected HEK-293 cells using an Axoclamp 200B amplifier (Axon Instruments) and low resistance pipettes (2-4 MΩ). The recordings were performed at 37 °C in an extracellular solution (EC) comprising (mM) 140 NaCl, 5 CsCl, 1.2 MgSO4, 1.2 NaH2PO4, 5 CaCl, 10 HEPES, 10 glucose, pH 7.4, and osmolality 290 mOsM. To block K+, Ca2+, Cl− and Na− ATPase currents we used Cs, nifedipine (20 μM), niflumic acid (30 μM), and ouabaine (1mM), respectively. The patch pipettes were filled with a solution comprising (mM) 100 Cs-glutamate, 1 MgCl, 10 HEPES, 2.5 Na2-ATP, 10 EGTA, CsCl2, pH 7.2, and osmolality 270 mOsM. In experiments to assess the effect of the optimized peptide we added 5 μM of either optimized - or scrambled control peptide to the internal solution. Liquid junction potential was calculated using pCLAMP 10 software (Molecular Devices) and corrected by 15 mV (Vmembrane = Vpipette - (15 millivolts (mV))). The cells were clamped to -43 mV (reversal potential) for 6 min to allow sufficient intracellular diffusion of both ions and peptides. The NCX current was recorded after a pre pulse protocol and immediate from -43 mV we elicited a descending voltage ramp from 120 mV to -100 mV. The NCX current was isolated using 5 μM Ni2+ added in the EC. The currents were normalized to the cell capacitance and the current (I) – voltage (V) relations were plotted from -100 to 100 mV.
**Densitometric analysis**—Densitometric analysis was performed using Image Gauge V4.0, ImageQuant TL (Amersham Biosciences) or Image J (USA).

**Statistics**—All data were expressed as mean ±SEM. Comparisons between two groups were analyzed using unpaired Student’s t-test (GraphPad software (Prism 5.04)). A p-value of <0.05 was considered statistically significant.
RESULTS

Confirmation of the PLM-NCX1 interaction—Several experiments were performed to confirm the PLM-NCX1 interaction. First, PLM was observed together with NCX1 in membrane fractions isolated from rat neonatal cardiomyocytes (Fig. 1A, left panel) and rat left ventricle (LV) (right panel) using specific PLM (epitope mapped in Fig. 1B) and NCX1 antibodies (32). Second, immunoprecipitation of endogenous NCX using anti-NCX1 in rat LV confirmed co-precipitation of endogenous PLM by immunoblotting with anti-PLM (Fig. 1C). Immunoprecipitation of exogenous NCX1 in lysate from HEK293 cells co-transfected with either PLM or PLM (S68D) (mimicking phosphorylated pSer68-PLM) revealed that both PLM and PLM (S68D) precipitated equivalently with NCX1 (Fig. 1D). Finally, pull-down assays using biotinylated peptides, covering the cytoplasmic part of PLM and pSer68-PLM (Fig. 1E, schematic illustrated in upper panel, immunoblotting analysis shown in lower panel), with recombinant His-TF-NCX1 cyt, containing the cytoplasmic part of NCX1, were performed with anti-biotin-agarose. Consistent with previous studies, His-TF-NCX1 cyt precipitated with both PLM (38-72) and pSer68-PLM (38-72) (Fig. 1F). The results confirmed that the cytoplasmic part of PLM binds directly to the cytoplasmic part of NCX1 and that the binding is independent of phosphorylation at serine 68. Sequence alignments show that the cytoplasmic part of PLM was identical in human, rat, mouse, pig and dog, except for a serine at position 69 in mouse (Fig. 1G).

Identification of the reciprocal PLM-NCX1 binding sequences—To map PLM binding sites in NCX1 more precisely, biotin-PLM cyt and biotin-pSer68-PLM cyt were used in the overlay assays with the membranes containing overlapping 20-mer peptides covering the PLM binding part of NCX1 (11). The two peptides showed a similar binding pattern and bound to PASKT- and QKHPD-containing sequences in NCX1 (Fig. 2A-B, marked in bold letters) which are consistent with the previous report (33). Biotin-PLM cyt and biotin-pSer68-PLM cyt bound also to NCX1 sequences containing the inhibitory XIP region (8) (Fig. 2A and B, underlined sequences). As peptides can be synthesized with a different yield, depending on their properties (hydrophobicity and confirmation) (34), the four NCX1 peptides covering amino acids 261-280, 267-286, 324-343, and 333-352 (black arrows in Fig. 2A-B) were also synthesized as purified biotinylated peptides in solution (schematic illustrated in Fig. 2C). These synthesized peptides were further analysed for their binding affinity to PLM expressed in HEK293. Pull-down assays using anti-biotin conjugated beads and immunoblotting with anti-PLM revealed that all four NCX1 peptides precipitated PLM with similar levels (Fig. 2D). Beads without any peptide were used as negative control.

Reciprocally, to map where the NCX1-PASKT- and NCX1-QKHPD-containing sequences bound in PLM, equal amount of biotin-NCX1(267-286), bitoin-NCX1(324-343) and biotin-NCX1(333-352) (peptides with bold amino acids in Fig. 2C) were used in the overlay assays on the membranes containing overlapping 20-mer peptides covering the cytoplasmic part of PLM and pSer68-PLM. The binding sites were detected by using anti-biotin-HRP antibody and the results revealed that all three NCX1 peptides bound to the same sequence in PLM; (Fig. 2E, RCKFNQQQRTGEPDEEGTF, upper panel). The binding pattern was identical in pSer68-PLM, indicating that the binding does not depend on the phosphorylation of PLM at serine 68 (Fig. 2E, lower panel).

Optimization of PLM binding sequences by two-dimensional peptide arrays— The two uppermost peptides in Fig. 2C, were excluded for further optimization as they contained part of the XIP region, which is known to be an autoregulatory domain of NCX1 binding to NCX1 cytoplasmic loop and to calmodulin (8). Thus, the two lowermost NCX1-(Q)KHPD containing sequences were further optimized by two dimensional peptide arrays where each amino acid in the native peptide sequences were systemically substituted with every possible amino acid. Immunoblotting using anti-biotin-HRP showed that substitutions in NCX1 (333-352) (KHPDKEIEQLIELANYQVLS) strongly increased binding to biotin-pSer68-PLM (Fig. 3A), but not in NCX1 (324-343) (ARILKEKQKHPDKEIEQLI, data not shown). The pSer68-PLM binding affinity was dramatically increased when the NCX1 (333-352) (KHPDKEIEQLIELANYQVLS) was substituted in position 1-11 (labelled with red circles) compared to internal control peptides (white circles). Especially, tryptophan (W) and tyrosine (Y) substitutions highly increased the affinity for pSer68-PLM (Fig. 3A, lower left square bracket).
Substitutions with negatively charged amino acids such as aspartic acid (D) and glutamic acid (E) were not favorable as they mostly reduced or abolished pSer68-PLM binding (Fig. 3A, upper left square bracket). Consistent with the disfavor for the negatively charged amino acids, substitutions of aspartic acid in position 4 (D4) and glutamic acid in position (E6) in the native NCX1 sequence mostly increased pSer68-PLM binding (indicated with arrows above the array in Fig. 3A).

In the second peptide membrane synthesis, the native NCX1 (333-352) peptide was single substituted with a tyrosine (Y) in position K1, H2, P3, K5, I7 or I11. Immunoblotting using anti-biotin-HRP antibody revealed that K1Y, H2Y, P3Y, and K5Y greatly increased the binding affinity to biotin-pSer68-PLM (Fig. 3B). In an attempt to increase the affinity to an even higher level, we performed double substitutions of the NCX1 (333-352) peptide. We combined each of the single mutations above with various amino acid substitutions in the flexible positions D4 and E6 (indicated by arrows above the array in Fig. 3A). The double mutation; K1Y, D4Y, increased the binding affinity to pSer68-PLM (Fig. 3B, labeled in red circles) compared to that of single mutated K1Y. Most of the other double substituted peptides showed an increased pSer68-PLM binding affinity compared to that of the native NCX1 peptide (Fig. 3B-G). In conclusion, pSer68-PLM binding was increased 8-fold when lysine (K) in position 1 and aspartic acid (D) in position 4 were substituted with tyrosines (K1Y, D4Y). These two positions correspond to K333 and D336 in the full length NCX1 protein (K333Y, D336Y) (numbering includes the signal peptide at N-terminus).

The optimized peptide binds strong to pSer68-PLM and block binding of NCX1-PASKT- and NCX1-KHPD — Pull down experiments using three biotinylated peptides; the NCX1 native sequence encompassing amino acids 333-352, the optimized sequence (K333Y, D336Y) and a scrambled sequence (schematic illustrated in Fig. 4A), together with an untagged PLM34 peptide were performed using anti-biotin agarose beads. Immunoblotting with anti-PLM showed that the optimized peptide bound stronger to PLM34 compared to the native NCX1 peptide sequence (Fig. 4B). Beads without presence of any peptide and the scrambled control peptide were used as negative controls. Consistent with the pull-down assays, the optimized peptide bound much stronger to PLM and pSer68-PLM synthesized on membrane, compared to that of native NCX1 (333-352) in the overlay assays (Fig. 4C, middle panels). In addition, the optimized peptide appeared to bind pSer68-PLM/PLM in a broader manner compared to that of native NCX1 (333-352) and it recognized the α-helical region in PLM (35) (sequences in bold in Fig. 4C).

To test if the optimized peptide was able to block NCX1 binding, an untagged version of the optimized peptide was pre-incubated with PLM34 and pSer68-PLM34 synthesized on membranes, before performing overlay assays with biotin-NCX1(267-286) (containing the PASKT motif) and biotin-NCX1(333-352) (containing the KHPD motif). Immunoblotting with anti-biotin-HRP showed that the optimized peptide efficiently blocked binding of NCX1-PASKT and NCX1-KHPD to pSer68-PLM/PLM (middle panels in Fig 4D and E, respectively). Altogether, our data indicate that the optimized peptide is as a prominent blocking peptide for the NCX1-PLM interaction.

Sequence alignments show that the native NCX1 sequence; KHPDKEIEQQLIELANYQVLS, and surrounding regions were almost identical in human, rat, mouse and dog, suggesting the optimized peptide is able to affect the NCX1-PLM interaction across species (Fig. 4F).

The optimized peptide reverses PLM (S68D) inhibition of NCX1 activity — To study the effect of the optimized peptide on NCX1 current in presence of pSer68-PLM, we transfected HEK293 cells with NCX1 and PLM (S68D) (mimicking phosphorylated PLM). HEK293 cells are devoid of PLM and NCX1 and have been shown to be a valid cell system to study isolated NCX1 current since they are electrically silent (36). The currents were recorded in whole cell patch clamp configuration and acquired using ramp protocol from 120 to -100 mV. The Ni2+ sensitive currents, corresponding to the isolated NCX1 current, were plotted as an I-V curve from -100 to 100 mV. The assays to measure the effect of PLM (S68D) were first validated without the presence of the optimized peptide. The normal NCX1 shown in Fig. 5 (black squares) had a robust current in both forward and reverse mode. Consistent with literature (13), co-transfecting NCX1 with PLM (S68D) inhibited both forward and reverse mode (brown triangles). The presence of the optimized peptide relieved the PLM inhibition (blue circles) and the NCX1 current returned to the range observed in NCX1 transfected cells without PLM (S68D). Contrary, the presence of a scramble peptide (control) did not relieve the inhibition of
phosphorylated PLM (black inverted triangles), strongly indicating that the reversal of NCX1 inhibition was sequence specific.

A model of pSer68-PLM inhibition of NCX1 activity and relieve of its inhibition by the optimized peptide is summarized in Fig. 6.
**DISCUSSION**

Although there are several reports suggesting that PLM is an endogenous NCX1 inhibitor (28), other groups have neither observed the direct effect of PLM on NCX1 activity nor the direct interaction between NCX1 and PLM (29,37,38).

Here we show that pSer68-PLM/PLM interacts directly with NCX1 and modulates NCX1 activity when PLM is phosphorylated at serine 68. Consistent with previous reports (13,16), we found that 1) both endogenous and PLM precipitated with NCX1 in HEK293 cell lysates and rat LV lysates. 2) Cytoplasmic part of pSer-68-PLM/PLM bound directly to the recombinant NCX1 cytoplasmic part. 3) Peptide array analyses confirmed that pSer68-PLM/PLM bound directly to PASKT- and QKHPD- containing NCX1 sequences, as previously reported (17,33). 4) Reciprocally, PASKT- and QKHPD- containing NCX1 sequences bound directly to the cytoplasmic part and the binding did not depend on serine 68 phosphorylation. 5) Finally, we developed a blocking peptide specific for the pSer68-PLM-NCX1 interaction which reversed the inhibitory effect of PLM (S68D) on NCX1 activity in HEK293 cells.

**Mapping of the PLM-NCX1 reciprocal interaction sites**—By extensive use of peptide array technology, we were able to map pSer68-PLM/PLM binding at the amino acid level in NCX1. PLM/pSer68-PLM bound to two regions in NCX1, containing the PASKT- and QKHPD motifs, which previously have been shown to be involved in PLM binding (17). PLM/pSer68-PLM bound also to the NCX1-XIP domain which is in close vicinity to the NCX1-PASKT motif. The XIP domain, containing a calmodulin binding site (39), has an autoregulatory role in regulating NCX1 activity (8) and is involved in Na+ dependent inactivation (40). PLM is also able to inhibit NCX1 when the XIP region is deleted (17).

NCX-PASKT and NCX1-QKHPD containing sequences bound to the N-terminus of the cytoplasmic part of PLM (RCKFNNQQRTGEPDEEEGTF). The NCX1 binding site resided outside serine 63, serine 68 and threonine 69, thus confirming that PLM-NCX1 interaction is not dependent on the PLM phosphorylation. The finding of an identical binding motif for NCX-PASKT and NCX1-QKHPD in PLM, suggests that the two motifs bind to each monomer in the PLM dimer. The two motifs are important for PLM regulation of NCX, because alanine mutations in these two motifs abolish or reduce NCX1 inhibition. Importantly, alanine mutation of lysine (K) in the QKHPD motif (QAHPD) completely removes PLM inhibition of NCX1 (33). Consistently, pSer68-PLM was not able to bind to NCX1 (333-352) on array, when the lysine (K) at position 333 was mutated to an alanine (Fig. 3A, AHPDKIEIEQLIELANYQVLS).

The NCX-PASKT and NCX1-QKHPD motifs are located within the catenin-like domain of NCX1 (CLD) (41). CLD is suggested to be important for transducing signals from the two Ca2+-binding domains in NCX1 (CBD1 and CBD2) to the transmembrane segments upon Ca2+ binding (41). It is less likely that PLM inhibits Ca2+- dependent activation of NCX1, since PLM is still able to inhibit NCX1 lacking CBDs (11) and the NCX1-G503P mutant, which lacks Ca2+ dependent activation of NCX1 (42). Therefore, it has been suggested that PLM might regulate NCX1 activity by altering oligomerization state of NCX1 (33). Experiments using chemical cross-linking (43) and fluorescence resonance energy transfer (44) have suggested that NCX1 exists as a dimer in the cell membrane of insect and Xenopus oocytes. Further experiments are required to investigate whether the NCX-PASKT and NCX1-QKHPD motifs are held distant or in close proximity in CLD, or in a possible dimer, and the role of PLM binding. The stoichiometry of interaction of PLM and NCX1 is unknown.

**Development of a High Affinity Phospholemman Binding Peptide**—The high affinity peptide was derived from the PLM binding sequence in NCX1, partially covering the QKHPD motif, which has been reported to interact with PLM (33). The optimization of the NCX1 derived sequence by using two-dimensional peptide arrays identified amino acids important for the enhancement of pSer68-PLM affinity and these amino acids were therefore substituted into specific positions into the sequence. The final optimized peptide; double substitution with tyrosine (YHPYKEIEQLIELANYQVLS) had approximately an 8-fold higher affinity for pSer68-PLM compared to the native NCX1 sequence. The optimized peptide was able to block binding of both NCX1-PASKT and NCX1-QKHPD to PLM and
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reverse the inhibitory effect of PLM (S68D) on NCX1 activity (both forward and reverse mode) in HEK293 cells. The reversal was specific because a scrambled control peptide had no effect.

Will the optimized NCX1 derived peptide interfere with the NKA-PLM interaction?—The regulatory effect of PLM on NCX1 and NKA are indeed distinctive. The cytoplasmic part of the PLM dimer binds to NCX1, whereas the transmembrane (TM) domain of PLM monomer binds to TM2, TM6 and TM9 of the α-subunit of NKA. Hence, phosphorylation of PLM at serine 68 rather relieves its inhibition of NKA (28). Phosphorylated PLM remains physically associated with NKA (29,45). Two PLM interaction sites in NKA have been reported (46). The functional interaction sites in PLM and NKA have been reported to be PLM-F28 and NKA-E960, respectively and these two sites are critical for effect of PLM on NKA. The second non-regulatory PLM-NKA interaction site has been suggested to be close to the N-terminus (extracellular) or within the TM domain of PLM. Since the NKA interactions are outside the cytoplasmic part of PLM, the optimized peptide (PLM binding peptide) will probably not interfere with the PLM-NKA interaction. Recently, PLM has been shown to modulate the activity of L-type Ca\(^{2+}\) channels in heterologous expression systems (20,47), but the association site(s) has not been identified.

Is reversal of the inhibitory effect of pSer68-PLM on NCX1 activity beneficial in HF?— PLM is considered as a stress protein (28). Under resting conditions, PLM is functionally quiescent and the in vivo myocardial contractility is similar between WT and PLM knock out mice (28,48). However, under catecholamine stress, when PLM is phosphorylated, one of its major physiological functions is to suppress Na\(^+\) overload through NKA activation. Maintaining low intracellular Na\(^+\) levels facilitates Ca\(^{2+}\) extrusion by NCX, it is predominant in “forward” mode of operation. However, such indirect effects of catecholaminergic stress on Ca\(^{2+}\) extrusion appear to be tempered by direct inhibition of NCX activity upon binding of phosphorylated PLM. Thus, the net effects of PLM on cardiomyocyte Ca\(^{2+}\) handling and contraction/relaxation are complex, particularly since experimental data indicate that NKA sets local gradients of Na\(^+\), which regulate NCX activity (49,50). Such insight suggests that therapeutic modulation of PLM levels may be less appropriate than modulation of PLM interaction with either NKA or NCX. Our data indicate that blockade of the interaction between pSer68-PLM and NCX increases NCX activity. Since NCX competes with SERCA, an increase in NCX activity has been shown to reduce sarcoplasmic reticulum Ca\(^{2+}\) content, and thus contractility (51). Thus, inhibition of the pSer68-PLM-NCX interaction would not be expected to be beneficial in the setting of systolic heart failure where contractility is depressed. However, during diastolic heart failure (Heart Failure with Preserved Ejection Fraction, HFPEF), reducing the Ca\(^{2+}\) store is of less concern and enhancing Ca\(^{2+}\) extrusion by NCX may favorably improve diastolic function in these patients.

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Abbreviations —NCX, Na\(^+\)-Ca\(^{2+}\) exchanger; HF, heart failure; LV, left ventricle; FL, full length; TMs, transmembrane segments; CBD, Ca\(^{2+}\) binding regulatory domain; CLD, catenin-like domain; XIP, exchanger inhibitory peptide; HEK, Human Embryonic Kidney; WT, wild type; PKCα, protein kinase C α; IP, immunoprecipitation
FIGURE LEGENDS

FIGURE 1 Confirmation of a direct PLM-NCX1 interaction. (A) NCX1 and PLM were analysed in the cytoplasmic and membrane fractions isolated from rat neonatal cardiomyocytes and LV lysates using NCX1 and PLM antibodies. GAPDH and calcequestrin were used as controls indicating cytoplasmic and membrane fractions, respectively. (B) Epitope mapping was performed by overlaying an array of immobilized overlapping 20-mer PLM peptides with anti-PLM (ab76597, left panel). Amino acids in bold were indicative for anti-PLM binding. Immunoblotting by omitting anti-PLM was used as a negative control (right panel). (C) Immunoprecipitation of rat LV lysates and (D) cell lysates from HEK293 cells co-expressing NCX1 and PLM or NCX1 and PLM (S68D), using anti-NCX1. Immunoprecipitates and lysates (as input) were visualized by immunoblotting using NCX1 and PLM antibodies. A specific anti-NCX1 blocking peptide and non-relevant rabbit IgG were used as negative controls in C. (E) Schematic presentation of biotinylated peptides covering PLM\textsubscript{cyt} and pSer68-PLM\textsubscript{cyt} (upper panel). The \(\alpha\)-helical region is indicated. Immunoblotting analysis of the two biotinylated peptides using anti-biotin-HRP is shown in the lower panel. (F) Pull down assay with biotin-PLM\textsubscript{cyt} and biotin-pSer68-PLM\textsubscript{cyt} against recombinant HIS-TF-NCX1\textsubscript{cyt} (containing the cytoplasmic part of NCX1) using monoclonal anti-biotin conjugated beads. Pull-down of NCX1 was analysed by immunoblotting using anti-NCX1. (G) Alignment of the cytoplasmic part of PLM in human, rat, mouse, pig and dog. Black boxes indicate the identical amino acids (DNA Star, Madison, Wisconsin).

FIGURE 2 Identification of pSer68-PLM/PLM binding in the cytoplasmic part of NCX1. (A) Biotin-PLM or (B) Biotin-pSer68-PLM binding was identified by overlaying the two biotinylated peptides on the membranes containing 20-mer overlapping NCX1 peptide sequences, followed by immunoblotting using anti-biotin-HRP (upper panels). The PLM binding residues, PASKT and QKHPD, identified by Zhang et al 2011 are marked in bold (33). Underlined amino acids indicate XIP region. Arrows indicate the four sequences that were investigated further. Membranes incubated with only anti-biotin-HRP were used as negative controls (lower panels in A and B). (C) Schematic illustration of four biotinylated NCX1 peptides used in pull-down assay. (D) Pull-down assay with the biotinylated NCX1 peptides against PLM expressed in HEK293 cells. PLM binding was analysed by immunoblotting using anti-PLM (upper panel). Beads were used as a negative control. A bar graph represents relative PLM binding quantified by densitometry analysis. Differences were analysed using unpaired t-test (**p<0.01, ***p<0.001) (n=4-7). Error bars represent standard error of the mean (SEM). (E) Overlay assays of three bionylated peptides; biotin-NCX1 (267-286) (PASKT), biotin-NCX1 (324-343) (QKHPD) and biotin-NCX1(333-352) (KHPD) on two membranes containing 20-mer overlapping PLM (upper panels) or pSer68-PLM (lower panels) peptide sequences, followed by immunoblotting using anti-biotin-HRP. Phosphorylated serine 68 is underlined. Negative controls were performed by omitting incubation of the biotinylated peptides with the membranes (right panels).

FIGURE 3 Optimization of pSer68-PLM binding sequences. (A) A two dimensional peptide array of NCX1 (333-352) (KHPDKKE1EQLIELANYQVLS) was synthesized and overlayed with biotin-pSer68-PLM. The binding was detected by using anti-biotin-HRP. Each residue in the native NCX1 sequence is written as a single-letter code above the array, whereas substitutions are given as single-letter codes to the left (vertically). The first row of the array shows the binding of biotin-pSer68-PLM to the native NCX1 (333-352). White circles indicate internal control peptides of the native sequence within the array. Red circles indicate substitutions that enhanced biotin-pSer68-PLM binding. Arrows above the array indicate the important positions; 4 and 6 in the native peptide sequence. The representative data were acquired from two independent experiments. (B-E) Biotin-pSer68-PLM binding to native, single and double substituted NCX1 (333-352) was analysed by immunoblotting using anti-biotin-HRP (lower panel). Relative pSer68-PLM binding was quantified by densitometry analysis (upper panel). The presented data is an average from two independent experiments. Red circle indicates the final optimized peptide in B.

FIGURE 4 Analysis of the optimized peptide sequence. (A) Schematic illustration of three biotinylated peptides; biotin-NCX1(333-352) (native sequence), biotin-NCX1(K333Y, D336Y) (optimized peptide sequence), biotin-NCX1-scrambled (control sequence) and the untagged PLM\textsubscript{cyt}.
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peptide used in pull down assay in B. (B) Pull-down assays with biotin-NCX1(333-352) and biotin-NCX1(K333Y, D336Y) (optimized peptide) against the untagged PLMcyt peptide. PLM binding was analysed by immunoblotting using anti-PLM. A biotin-NCX1 (333-352) scrambled peptide and beads were used as negative controls. (C) Binding sites of biotin-NCX1(333-352) and biotin-NCX1(K333Y, D336Y) (optimized peptide) to PLM and pSer68-PLM were identified by overlay assays on membranes containing 20-mer overlapping PLM (upper panels) and pSer68-PLM (lower panels) peptide sequences, followed by immunoblotting using anti-biotin-HRP. Binding of (D) biotin-NCX1 (267-286) and (E) biotin-NCX1(333-352) to PLM (upper panels) and pSer68-PLM (lower panels) with or without a pre-incubation of the optimized peptide. Binding was analysed by immunoblotting using anti-biotin-HRP. Phosphorylated serine 68 is underlined in C-E. Incubation with only anti-biotin-HRP (omitting incubation with the peptides) was used as negative control (right panels in C-E). (F) Alignment of human, rat, mouse and dog NCX1 sequence. Position of the XIP region and the native NCX1 sequence (amino acids 333-352) used for the optimization are underlined. Black boxes indicate identical amino acids (DNA Star, Madison, Wisconsin).

FIGURE 5 The optimized peptide reverses PLM (S68D) inhibition of NCX1. Whole cell patch clamp recordings in transfected HEK293 cells. The different current traces were evoked using a voltage ramp from 120 to -100 mV. The Ni²⁺ sensitive currents were normalized to the cell capacitance (picoampere (pA)/picofarad (pF)). The I-V curves are plotted with the ordinate showing normalized current and the abscissa showing the voltage in mV. NCX1 alone (black squares, n=11), NCX1 co-expressed with PLM (S68D) (brown triangles, n=4), NCX1 co-expressed with PLM (S68D) and added 5 μM of the optimized peptide (black inverted triangles, n=6), and NCX1 co-expressed with PLM (S68D) and added 5 μM of the scrambled control peptide (blue circles, n=6) (student t-test,* p<0.05).

FIGURE 6 A model of PLM (S68D) inhibition of NCX1 activity and relieve of its inhibition by the optimized peptide. (A) pSer68-PLM (phosphorylation denoted with a yellow star) binds to the cytoplasmic loop of NCX1 and inhibits NCX1 activity (16,18). (B) The optimized peptide, derived from a pSer68-PLM binding sequence in NCX1, binds to cytoplasmic PLM causing dissociation of pSer68-PLM from NCX1, thus relieves the inhibitory effect of pSer68-PLM.
REFERENCES


Increased NCX1 activity by a Specific PLM Binding Peptide


Figure 2
Wanichawan et al.
Figure 3
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Figure 4
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Figure 5
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A

NCX1 activity is inhibited

B

Inhibition of NCX1 is relieved by the Opt-Pep

Figure 6
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