UNIVERSITY OF CINCINNATI

Date: 7-Oct-2010

I, Nabeel Almoamen, hereby submit this original work as part of the requirements for the degree of:
Doctor of Philosophy
in Molecular Genetics, Biochemistry, & Microbiology
It is entitled:
Role of AE3 Cl-/HCO3- Exchanger in the Development of Heart Disease

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Role of AE3 Cl⁻/HCO₃⁻ Exchanger in the Development of Heart Disease

A dissertation submitted to the
Graduate School
of the University of Cincinnati
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
(Ph.D.)

In the Department of Molecular Genetics, Biochemistry and Microbiology
of the College of Medicine

2010

by

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ABSTRACT

The AE3 Cl⁻/HCO₃⁻ exchanger is expressed abundantly in the sarcolemma of cardiomyocytes, where it mediates Cl⁻-uptake and HCO₃⁻-extrusion. Inhibition of AE3-mediated Cl⁻/HCO₃⁻ exchange has been suggested to protect against cardiac hypertrophy; however, other studies indicate that AE3 might be necessary for optimal cardiac function. To test these hypotheses and shed further light on the role of AE3 in the heart we crossed AE3-null mice, which appear phenotypically normal, with two distinct models of heart disease. The first model, Tm180, is a transgenic mouse model of hypertrophic cardiomyopathy (HCM), whereas the second model, Tm54, is a transgenic mouse model of dilated cardiomyopathy (DCM). A third model that was used for this investigation is a mouse model of heart disease induced by infusion of angiotensin II (AngII), which leads to cardiac hypertrophy and hypertension.

In the Tm180 HCM model, loss of AE3 had no effect on cardiac hypertrophy; however, survival of TM180/AE3 double mutants was sharply reduced compared with TM180 single mutants. Analysis of cardiac performance revealed impaired cardiac function in TM180 and TM180/AE3 mutants relative to wild-type (WT) mice, with double mutants being more severely affected and exhibiting little response to β-adrenergic stimulation. Phosphorylation of phospholamban on Ser16 was sharply increased in single and double mutants relative to wild-type hearts under basal conditions, leading to reduced reserve capacity for β-adrenergic stimulation of phospholamban phosphorylation. Imaging analysis of isolated myocytes revealed reductions in the amplitude and prolongation of the decay of Ca²⁺ transients in both mutants, with greater reductions in TM180/AE3 double mutants. Thus, loss of AE3 in the TM180 cardiomyopathy model had no apparent anti-hypertrophic effect but led to further impairment of
cardiac performance and Ca$^{2+}$ handling, loss of β-adrenergic stimulation of contractility and relaxation, and more rapid decompensation and heart failure.

With respect to the Tm54 model, the degree of cardiac hypertrophy was slightly less in Tm54/Æ3 double mutants than in Tm54 mutants; however, both Tm54 and Tm54/Æ3 mutants showed similar degrees of heart enlargement vs. WT and Æ3-null controls, indicating that the enlargement due to the Tm54 mutation was unaffected by the loss of Æ3. Levels of β–myosin heavy chain (β–MHC) were significantly increased in cardiac homogenates of both Tm54 and Tm54/Æ3 mice relative to WT samples, although a significantly lower level was observed in Tm54/Æ3 vs. Tm54. Analysis of heart function revealed severely depressed contractility and relaxation in both mutant genotypes when compared with WT mice, and there was no improvement in cardiac function in Tm54/Æ3 vs. Tm54 mice. In contrast, analysis of Ca$^{2+}$ transients in isolated cardiomyocytes revealed significantly higher amplitudes in Tm54/Æ3 cells vs. Tm54 or WT cells. These data indicate that the loss of Æ3 has a positive impact on calcium handling in Tm54 cardiomyocytes but does not improve the health of Tm54 mutants.

Finally, no beneficial effects of Æ3 ablation were observed in the third model, in which heart disease was induced by infusion of Ang II. The degree of cardiac hypertrophy and effects of AngII treatment on cardiac performance were the same in both WT and Æ3-null mice.

The overall conclusion of the research in this thesis is that ablation of Æ3 does not reduce the degree of cardiac hypertrophy and heart disease, at least in the models analyzed in this work, and that it leads to more rapid decompensation in an HCM model. Although there are some possible beneficial effects on Ca$^{2+}$ handling in the DCM model, the data suggest that Æ3 is unlikely to be an appropriate target for inhibitory drug treatment of heart disease.
Dedication

To my wife.
AKNOWLEDGMENT

My deep thanks and gratitude go to my Thesis Committee Members especially to my advisor Dr. Gary E. Shull. I thank him for all the encouragement and continuous support and especially I thank him for teaching me to do good science.

I thank our lab members: Dr. Vikram Prasad, Emily Bradford (former graduate student), Martha Jiang, Dr. Zachary Spicer, and Kani Vairamani. I am especially indebted to Vikram for his continuous technical support and his sincere commitment to helping me in my research.

My deep thanks go to Michelle Neiman and Valerie Lasko from Dr. John Lorenz’s laboratory in the Department of Molecular and Cellular Physiology for their excellent collaborations with our laboratory and their willingness to help in my studies.

I also present my thanks to Maureen Bender for her excellent animal husbandry and caring for the mouse colonies.

I forward my deep thanks to Dr. Ilona Bodi for her commitment in teaching me how to prepare cardiomyocytes and to perform calcium imaging studies.

My deep gratitude goes to Dr. Edmund Choi for his sincere help and encouragement during the first two years of my studies.

My thanks also go to the Fulbright Foundation and US Embassy staff in Bahrain for all their help and facilitation of my scholarship.

Also I present my thanks to the Ministry of Health in Bahrain for supporting my scholarship and especially I am indebted to our Pathology Chairperson at that time, Dr. Raja Al Yousuf, for her sincere enthusiasm to help.

Finally, I am especially grateful and give my deepest thanks to my family members for their continuous support and care. I am especially indebted to my wife for her sincere support and encouragement since day one of my scholarship. I especially thank her for taking care of the children during my long absence.
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LISTS OF ABBREVIATIONS

+\( \frac{dP}{dt} \): Maximal rate of contraction
\( \frac{dP}{dt_{40}} \): Maximal rate of contraction normalized for an after-load blood pressure of 40 mm Hg
-\( \frac{dP}{dt} \): Maximal rate of relaxation
[\( \text{Ca}^{2+} \)]i: Intracellular \( \text{Ca}^{2+} \) ion concentration
[\( \text{Na}^{+} \)]i: Intracellular \( \text{Na}^{+} \) ion concentration
\( \text{AR} \): Alpha-adrenergic receptor
AE1, 2 and 3: Anion exchanger isoform 1, 2, and 3
AE3c: Cardiac isoform of AE3
AE3fl: Full length isoform of AE3
AngII: Angiotensin II
ANP: Atrial (or type A) natriuretic peptide
\( \text{AR} \): Beta-adrenergic receptor
\( \text{MHC} \): Beta-myosin heavy chain
CamKII: Calcium/calmodulin-dependent protein kinase II
CICR: Calcium-induced calcium release
cTnC: Cardiac troponin C (calcium binding Tn)
cTnI: Cardiac troponin I (inhibitory Tn)
cTnT: Cardiac troponin T (tropomyosin binding Tn)
DCM: Dilated cardiomyopathy
DHPR: Dihydropyridine receptor
EC coupling: Excitation-contraction coupling
EF\%: Percent of ejection fraction
ERK: Extracellular signal-regulated kinase
FFR: Force-frequency relationship (or response)
FS\%: Percent of fractional shortening
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GPCR: G-protein coupled receptor
H/BW (HW/BW): Heart weight-to-body weight ratio
HCM: Hypertrophic cardiomyopathy
HF: Heart failure
HR: Heart rate
I/R injury: Ischemia-reperfusion injury
IACUC: Institutional animal care and use committee
ISO: Isoproterenol
KO: Knockout
LTCC: L-type calcium channel
LV: Left ventricle
LVEDD: Left ventricular end-diastolic dimension (or diameter)
LVESD: Left ventricular end-systolic dimension (or diameter)
LVM: Left ventricular mass
MAPK: Mitogen-activated protein kinase
mm Hg: millimeter mercury
NBCe1 (NBC1): The electrogenic sodium-bicarbonate cotransporter e1
NBCe2 (NBC4): The electrogenic sodium-bicarbonate cotransporter e2
NBCn1 (NBC2 or 3): The electroneutral sodium-bicarbonate cotransporter n1
NCX1: Sodium-calcium exchanger 1
NHE1: Sodium-hydrogen (or proton) exchanger 1
NKCC1: Sodium-potassium dichloride cotransporter 1
PAT1: Putative anion transporter 1
pHi: Intracellular pH
PKA: c-AMP-activated protein kinase A
PKC: Protein kinase C
PLN (or PLB): Phospholamban
PP1: Protein phosphatase type 1
PP1-c: Catalytic subunit of PP1
PP2A: Protein phosphatase type 2A
PP2A-c: Catalytic subunit of PP2A
$R_{340/380}$: Fluorescence ratio of excitation at 340 nm-to-excitation at 380 nm
**RyR2**: Ryanodine receptor 2  
**S-actin**: Sarcomeric actin  
**SERCA2a**: Sarco(endo)plasmic reticulum calcium ATPase 2a  
**Slc26**: Solute carrier family 26  
**Slc4**: Solute carrier family 4  
**SR**: Sarcoplasmic reticulum  
**Tg (or TG)**: Transgenic  
**Tm180**: Tropomyosin mutation at codon 180 (Glu-Gly) and the correspondent Tg mice  
**Tm180/AE3**: Double mutant mice having Tm180 and AE3-null genotype.  
**Tm54**: Tropomyosin Mutation at codon 54 (Glu-Lys) and the correspondent Tg mice  
**Tm54/AE3**: Double mutant mice having Tm54 and AE3-null genotype.  
**Tn**: Troponin  
**WT**: Wild-type
Chapter 1: INTRODUCTION

Overall Objectives

At least four electroneutral Na\(^+\)-independent Cl\(^-\)/HCO\(_3\)\(^-\) exchangers and four Na\(^+\)-dependent acid extruders are expressed in cardiac myocytes (Fig. 1.1). The Cl\(^-\)/HCO\(_3\)\(^-\) exchangers, which mediate HCO\(_3\)\(^-\) extrusion and Cl\(^-\) uptake and therefore tend to acidify the cell, include three anion exchangers (AEs) of the Slc4a gene family (AE1-3) and one member of the Slc26a gene family (PAT1 or Slc26a6). The Na\(^+\)-dependent acid extruders include the NHE1 Na\(^+\)/H\(^+\) exchanger and three Na\(^+\)-HCO\(_3\)\(^-\) cotransporters (NBCn1, NBC1, and NBC4) of the Slc4a gene family (Fig. 1.1). They utilize Na\(^+\)-uptake to drive either H\(^+\)-extrusion or HCO\(_3\)\(^-\)-uptake and can therefore alkalinize the cell. Although Cl\(^-\)/HCO\(_3\)\(^-\) exchangers and Na\(^+\)-dependent acid extruders operating alone can affect intracellular pH (pH\(_i\)), Cl\(^-\)/HCO\(_3\)\(^-\) exchange operating in concert with NHE1 or one of the NBCs can serve as pH\(_i\)-neutral Na\(^+\)-loading mechanisms.

![Figure 1.1](image.png)

**Figure 1.1.** The major acid loaders (AE1, AE2, AE3 and PAT1) and acid extruders (NHE1, NBCn1, NBC1 and NBC4) in cardiac myocytes [1]. NBCn1 is electroneutral, with cotransport of Na\(^+\) and HCO\(_3\)\(^-\) in a 1:1 ratio, whereas NBC1 and NBC4 are electrogenic, with cotransport of Na\(^+\) and HCO\(_3\)\(^-\) in a 1:2 ratio. Cl\(^-\)/HCO\(_3\)\(^-\) exchange operating in concert with either Na\(^+\)/H\(^+\) exchange or Na\(^+\)-HCO\(_3\)\(^-\) cotransport contributes to pH\(_i\)-neutral Na\(^+\)-loading, which can affect Ca\(^{2+}\)-loading via effects on Na\(^+\)/Ca\(^{2+}\) exchange (NCX) activity. Also shown is the NKCC1 Na\(^+\)-K\(^+\)-2Cl cotransporter, which also mediates pH\(_i\)-neutral Na\(^+\)-loading.
Na\(^+\)-loading in cardiomyocytes can occur by a variety of mechanisms [2], including NHE1-mediated Na\(^+\)/H\(^+\) exchange, Na\(^+\)-HCO\(_3\)\(^-\) cotransport, and NKCC1-mediated Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport, as illustrated in Fig. 1.1, and other mechanisms. Na\(^+\)-loading is known to affect cardiac function through its effects on Ca\(^{2+}\) loading. When intracellular Na\(^+\) concentrations increase, particularly in subsarcolemmal microdomains, it reduces the rate of Ca\(^{2+}\) extrusion via the NCX1 Na\(^+\)/Ca\(^{2+}\) exchanger (encoded by \textit{Slc8a1}), and because NCX1 is electrogenic (3 Na\(^+\):1 Ca\(^{2+}\)), increased intracellular Na\(^+\) can contribute to reversal of the exchanger and subsequent Ca\(^{2+}\)-uptake at depolarizing membrane potentials. It has been suggested that the AE3 Cl\(^-\)/HCO\(_3\)\(^-\) exchanger, the most abundant cardiac-specific of the Cl\(^-\)/HCO\(_3\)\(^-\) exchangers in cardiac myocytes (discussed below), works in concert with Na\(^+\)-dependent acid extruders, particularly the NHE1 Na\(^+\)/H\(^+\) exchanger, to increase intracellular Na\(^+\) while maintaining pH\(_i\) [3]. Thus, AE3 has been proposed to play a major role in pH\(_i\)-neutral Na\(^+\)-loading in cardiomyocytes. As such, it could have a major effect on cardiac performance in health and disease.

The primary objective of this thesis research is to better understand the functions of the AE3 Cl\(^-\)/HCO\(_3\)\(^-\) exchanger, both in the normal heart and particularly in disease conditions. It has been suggested that inhibition of AE3 activity might have potential therapeutic benefits in heart disease involving hypertrophy, arrhythmias, and ischemia [4]; however, there have been no definitive analyses of the role of AE3 in cardiac tissue subjected to disease conditions. To address this issue, an AE3-null mouse model prepared previously in the lab was crossed with two different transgenic cardiomyopathy models involving \(\alpha\)-tropomyosin: a Glu180Gly mutant that causes hypertrophic cardiomyopathy and increased Ca\(^{2+}\) sensitivity of the myofibrillar apparatus and a Glu54Lys mutation that causes dilated cardiomyopathy and decreased Ca\(^{2+}\) sensitivity of the myofibrillar apparatus. Additional studies, including the effects of angiotensin-induced
hypertrophy and an analysis of Ca\(^{2+}\) handling in AE3-null cardiomyocytes were also performed. The data indicate that AE3-mediated Cl\(^{-}\)/HCO\(_3^{-}\} exchange is required for compensation during heart failure, and that the absence of its activity can lead to decompensation and death.

**The Na\(^{+}\)-Independent Cl\(^{-}\)/HCO\(_3^{-}\} Exchanger AE3**

Anion exchanger isoform 3 (AE3) is an integral membrane protein and, as noted above, is a member of the *Slc4a* family of bicarbonate transporters that includes both Cl\(^{-}\)/HCO\(_3^{-}\} exchangers and Na\(^{+}\)-HCO\(_3^{-}\} cotransporters. There are at least 10 different members of the *Slc4a* family, each encoded by separate genes, and multiple protein variants for each gene arise by means of alternative promoters and alternative splicing [5]. AE3, the major cardiac isoform, is closely related in both structure and function to two other members of the *Slc4a* family that are expressed in heart, namely AE1 and AE2. These anion exchangers exhibit an overall amino acid sequence identity of 53-56% [5] and are encoded by 3 distinct genes: *Slc4a1* (encoding AE1), *Slc4a2* (AE2), and *Slc4a3* (AE3). General features of the 3 members of this sub-family that are expressed in heart are presented in Table 1. Each transporter mediates electroneutral anion exchange activity, with extracellular chloride being exchanged for intracellular bicarbonate. As such they function as bicarbonate extruders, i.e., displaying an acid-loading activity in almost all tissues. The one exception is AE1 which, because of the unusual driving forces, mediates the reverse activity in the lung. Hence, in most tissues these proteins play a pivotal role in the regulation of intracellular pH (pH\(_i\)) as well as intracellular chloride concentrations [Cl\(^{-}\)]\(_i\) and cell volume [6-11].
Table 1.1. General features of the AE1, 2 and 3 family members of the SLC4 anion transporters (table adapted from Ref [5, 11]).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Protein name</th>
<th>Tissue distribution</th>
<th>KO mice phenotype</th>
<th>Human gene locus</th>
<th>Gene Sequence accession #</th>
<th>Splice variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC4A1</td>
<td>AE1</td>
<td>Red blood cells, kidney, heart</td>
<td>Hemolytic anemia</td>
<td>17q21-d22</td>
<td>NM_000342</td>
<td>2</td>
</tr>
<tr>
<td>SLC4A2</td>
<td>AE2</td>
<td>Near ubiquitous</td>
<td>Stomach achlorhydria, failed dentition</td>
<td>7q35-q36</td>
<td>NM_003040</td>
<td>5</td>
</tr>
<tr>
<td>SLC4A3</td>
<td>AE3</td>
<td>Brain, retina, heart</td>
<td>Retinal defect, Agent-induced seizure sensitivity, Cardiac force-frequency defect*</td>
<td>2q36</td>
<td>NM_005070</td>
<td>≥2</td>
</tr>
</tbody>
</table>

* V. Prasad et al., unpublished observations
Tissue localization studies show high expression of AE3 mRNA transcripts in brain and heart, with heart expressing a truncated cardiac-specific variant (AE3<sub>c</sub>; 1030 amino acids) versus the full-length variant (AE3<sub>f</sub>; 1227 amino acids) that was first identified in brain, stomach, and retinal tissues [12-14]. These variants result from alternative promoter usage of the same gene [12, 13]. Immunoblot analysis using an AE3-specific antibody [4] and AE3-null tissues as negative controls allowed me to confirm the relatively limited tissue distribution of AE3 (Fig. 1.2). As expected, expression was very high in heart and brain, with low expression in stomach, and little, if any, expression in other tissues (Fig. 1.2).

**Figure 1.2.** Western blot analysis showing AE3 protein levels in different tissues. Tissues from AE3-null mice are included as controls for nonspecific binding of the antibody. Note that the prominent bands in liver, as well as other smaller bands in several other tissues, are nonspecific. GAPDH was used as loading control.
So far, a high resolution structure has not been published for AE3 or any of the anion exchangers; however, a topology model based on hydropathy analysis and partial X-ray crystallography of AE1 suggested a model with 14-transmembrane or membrane-embedded domains and at least two re-entrant loops in the lipid bilayer [6]. Both the amino- and the carboxyl-terminal ends are cytosolic, and an N-glycosylation site occurs in the third extracellular loop [15] (Fig. 1.3). In AE3\(_{\text{fl}}\), the N-terminal cytosolic domain spans approximately 700 amino acids. In AE3\(_{c}\), the first 270 amino acids of AE3\(_{\text{fl}}\) is replaced by an alternative 73 amino acids encoded by an alternative cardiac-specific first exon that occurs in intron 6 of the AE3\(_{\text{fl}}\) primary transcription unit.

Figure 1.3. Predicted topology model of AE3 (adapted from Reference [9]). As discussed in the text, the protein contains an extended N-terminal cytosolic domain that differs between the full length form (AE3\(_{\text{fl}}\)) found in brain and several other tissues and the truncated cardiac-specific form (AE3\(_{c}\)). It also contains several potential re-entrant loops and as many as 14 transmembrane helices that are responsible for the anion exchange functions of the protein, and it has a short cytosolic C-terminal domain.
With regard to possible functions of AE3, regulation of pH\textsubscript{i} is of utmost importance for cells functioning with high metabolic or energy demands, such as cardiac myocytes. In cardiac muscle, continuous cycles of contraction and relaxation lead to the production of high levels of CO\textsubscript{2} as a product of cellular respiration. Subsequent acid-loading occurs via carbonic anhydrase-facilitated reaction of CO\textsubscript{2} and H\textsubscript{2}O to produce carbonic acid, with dissociation to produce H\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-}. The need for robust pH\textsubscript{i} regulation is particularly important in disease conditions that cause gross pH\textsubscript{i} perturbations, as in the case of hearts undergoing ischemic episodes [16]. Intracellular acidity is increased in affected cardiomyocytes (lower pH\textsubscript{i}), due in part to the failure or inadequacy of acid-removal processes. Thus, it is obvious that a robust system is needed to maintain physiological acid-base homeostasis for such demanding cells. A number of transporters have been reported, or suggested, to be involved in such acid-base regulation, including AE3 and the other anion exchangers and Na\textsuperscript{+}-dependent acid extruders shown in Fig. 1.1. Indeed, stilbene derivatives, which function as relatively non-specific inhibitors of HCO\textsubscript{3}\textsuperscript{-} transporters, tend to reverse the effects of ischemia-associated pH\textsubscript{i} perturbations [17]. Given the high expression of AE3 in heart (see Fig. 1.2 and discussion below), AE3 would appear to be a reasonable candidate for such a therapeutic inhibitory effect of stilbene derivatives. Moreover, modulation of pH\textsubscript{i} homeostasis is associated with regulation of Ca\textsuperscript{2+} homeostasis in cardiomyocytes, and has been reported to affect myofilament Ca\textsuperscript{2+} sensitivity, with subsequent direct effects on contractility [18, 19].

Another interesting possibility regarding the function of AE3 in heart is its hypothetical role in the development of cardiac hypertrophy due to coupling with Na\textsuperscript{+}-dependent acid extruders, such as the NHE1 Na\textsuperscript{+}/H\textsuperscript{+} exchanger [3, 20]. Pathological cardiac hypertrophy is considered as a preliminary stage leading to overt heart failure. NHE1 has been shown
previously to play a major role in the progression of cardiac hypertrophy [3]. Increased NHE1 activity leads to an increase in intracellular Na\(^+\). This in turn leads to inhibition of the forward mode of the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1), which mediates the exchange of 3 Na\(^+\) and 1 Ca\(^{2+}\) (Fig. 1.1). With normal electrochemical gradients (low intracellular Na\(^+\)) this coupling ratio favors the extrusion of Ca\(^{2+}\). Inhibition of the exchanger occurs when intracellular Na\(^+\) accumulates; in addition, increased intracellular Na\(^+\) causes Ca\(^{2+}\)-loading by stimulating the reverse mode of NCX1. It has been suggested that AE3, working in concert with NHE1 to maintain neutral pH\(_i\), may allow an increase in NHE1 activity, with subsequent intracellular Na\(^+\)-loading [20]. By providing Cl\(^-\)/HCO\(_3\)^- exchange activity that could balance, and thus enhance, NHE1-mediated Na\(^+\)/H\(^+\) exchange, AE3 was suggested to be involved in this hypertrophic cascade, which might also involve a protein kinase C (PKC) signaling cascade [20, 21].

AE3-null mice were generated several years ago in our laboratory. Null mutants were born in normal Mendelian ratios and appeared to be healthy [1]. In addition, analysis of \textit{in vivo} cardiac function by use of intraventricular pressure measurements in anesthetized closed-chest mice revealed normal cardiac performance under both basal conditions and after \(\beta\)-adrenergic stimulation [1]. Other investigators have suggested that inhibition of AE3 might provide antiarrhythmic or other cardioprotective effects [4]; however, recent studies in our laboratory that have not yet been published show that AE3-null mice have a blunted force-frequency response (FFR) (Prasad V, unpublished observations). Also, a recent report pertinent to the effects of genetic ablation or inhibition of AE3 showed that AE3-null mice display a reduced seizure threshold in response to seizure-inducing agents when compared with wild-type mice [22]. AE3\(\beta\) is expressed at high levels in brain, so if inhibition of AE3 were to be developed as a cardioprotective strategy it would be necessary to avoid such adverse effects on neuronal tissue.
The Na⁺-Independent Cl-/HCO₃⁻ Exchanger PAT1

Another important Na⁺-independent Cl-/HCO₃⁻ exchanger in heart is putative anion transporter 1 (PAT1), which is a member of the Slc26a gene family (Slc26a6). PAT1 mediates electroneutral Cl⁻/HCO₃⁻ exchange; however, it also mediates chloride/formate exchange and some reports suggest it might function as an electrogenic Cl⁻/HCO₃⁻ exchanger (1Cl⁻/2HCO₃⁻) [11]. Preliminary data from our laboratory show that loss of PAT1 in mice has distinctly different effects on cardiac function than loss of AE3. Indeed, PAT1-null mice exhibit an increase in contractility after β-adrenergic stimulation (Prasad V., unpublished observations). However, the major phenotype for PAT1-null mice, which are relatively healthy, has been attributed to intestinal and renal functions relevant to Cl⁻/Oxalate exchange activity [23]. Although PAT1 is clearly a major Cl⁻/HCO₃⁻ exchanger in heart and could provide some degree of compensation in AE3-null hearts, this thesis research is restricted to analyses of the functions of AE3.

Sodium Handling in Cardiac Myocytes

Na⁺ handling (both influx and efflux) in cardiac myocytes involves a number of ion transporters and Na⁺ channels. The major driving force that moves Na⁺ across the cell membrane is the transmembrane electrochemical gradient. Sodium ion concentrations on the extracellular side of the membrane is ~140 mM, whereas intracellular Na⁺ is maintained at ~4-16 mM [24]. The principal pathway for Na⁺ extrusion is the Na⁺-K⁺-ATPase (sodium pump), which functions at the expense of ATP hydrolysis [2]. The sodium pump moves two potassium ions into the cell and extrudes three sodium ions. As such, this powerful pump maintains relatively constant sodium and potassium gradients across the cell membrane, which are used to drive changes in membrane potential during each heartbeat, and it also maintains the electrical gradient.
Voltage-sensitive sodium channels, the cardiac Na⁺/Ca²⁺ exchanger (NCX1), and other Na⁺ transporters mentioned below serve as major pathways for Na⁺ influx. In response to perturbations of cellular pH or volume homeostasis, either NHE1 or NKCC1 can be activated and contribute to Na⁺ influx. NHE1 plays a major role in H⁺ extrusion for pHᵢ control, whereas NKCC1 functions in regulation of cell volume through osmotic influx of H₂O accompanying uptake of Na⁺, K⁺, and Cl⁻ [24, 25]. NBC activity is also activated in cardiac myocytes in response to low pHᵢ, in which case it functions as an effective acid extrusion mechanism (inward movement of Na⁺ and HCO₃⁻) [7]. Consequently, these ion transport proteins play a major role in restoring the homeostatic state of the cell. Table 2 summarizes functional properties of the major ion transport proteins in sodium handling in the cardiac myocyte.

Table 1.2. Major sodium transport proteins in the cardiac myocyte [2].

<table>
<thead>
<tr>
<th>Ion Transport Protein</th>
<th>Nickname</th>
<th>Transport and Stoichiometry</th>
<th>Resting Flux Rate (mM/min)</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺/K⁺-ATPase</td>
<td>NKA</td>
<td>Na⁺ pump, I_pump</td>
<td>0.77</td>
<td>Cardiac glycosides</td>
</tr>
<tr>
<td>Na⁺ in-flux pathways</td>
<td></td>
<td>3 Na⁺/2 K⁺ exchange</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺ channel/current</td>
<td>IₙNa</td>
<td>Uncoupled</td>
<td>0.14</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>Na⁺/Ca²⁺ exchanger</td>
<td>NHE</td>
<td>2 Na⁺/1 Ca²⁺ exchange</td>
<td>0.28</td>
<td>Ni²⁺</td>
</tr>
<tr>
<td>Na⁺/H⁺ exchanger</td>
<td>NHE</td>
<td>1 Na⁺/1 H⁺ exchange</td>
<td>0.12</td>
<td>Amiloride, cariporide</td>
</tr>
<tr>
<td>Na⁺/HCO₃⁻ cotransporter</td>
<td>NBC</td>
<td>1 Na⁺/1 HCO₃⁻</td>
<td>0.12</td>
<td>DIDS, SITS</td>
</tr>
<tr>
<td>Na⁺/K⁺/2Cl⁻ cotransporter</td>
<td>NKCC</td>
<td>1 Na⁺/1 K⁺/2 Cl⁻ cotransport</td>
<td>0.15</td>
<td>Euxoside, bumetanide</td>
</tr>
<tr>
<td>Na⁺/Mg²⁺ antiporter</td>
<td>NaMgX</td>
<td>2 Na⁺/1 Mg²⁺ exchange</td>
<td>0.08</td>
<td>Imipramine</td>
</tr>
</tbody>
</table>

In addition, NCX1, NHE1, and NBC isoforms all contribute to Na⁺ handling and control of intracellular calcium levels [Ca²⁺], either directly through the NCX1 Na⁺/Ca²⁺ exchanger or indirectly via Na⁺-uptake by NHE1 and the different NBC isoforms. Interestingly, NCX1 can work in the reverse mode, i.e., extrusion of Na⁺ in exchange for Ca²⁺. Indeed, this condition is implicated in heart failure that is associated with elevated intracellular Na⁺ [26]. It is well recognized that calcium ions play a major role the regulation of heart function and tuning of the
excitation-contraction coupling cycle. Obviously, an elevation in intracellular Na\(^+\) will have a major impact on the intracellular Ca\(^{2+}\), which in turn causes an increase in cardiac contractility.

**Na\(^+\)-Dependent Acid-Base Transporters Functionally Relevant to AE3**

Important biological functions associated with modulation of intracellular Na\(^+\) in cardiac myocytes include pH\(_i\) regulation, regulation of excitation-contraction coupling, and regulation of cell growth. The correlation of changes in intracellular Na\(^+\) levels with heart abnormalities is widely appreciated. For example, the increase in intracellular Na\(^+\) concentrations in ischemia-reperfusion injury and the central role of pH\(_i\) in this pathological process are well established [3]. In addition, there is compelling evidence that impaired Na\(^+\) homeostasis contributes to other pathophysiological conditions such as cardiac hypertrophy and the associated heart failure [3].

Maintenance of Na\(^+\) homeostasis in the cardiac myocyte is a complex process and, as discussed above, involves the activities of a number of ion transporters. Given the critical role of Na\(^+\) in cardiac function, either directly or by its effects on Ca\(^{2+}\) homeostasis, it is important to understand the functions of the various Na\(^+\) transporters and their possible interactions with Cl\(^-\)/HCO\(_3^-\) exchangers such as AE3. Major Na\(^+\) handling proteins that may serve as important regulators of Na\(^+\) homeostasis in cardiac myocytes (see Fig. 1.1) include the following: Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1), the electrogenic Na\(^+\)-HCO\(_3^-\) cotransporter isoform 1 (NBC1 or NBCe1), the electroneutral Na\(^+\)-HCO\(_3^-\) cotransporter (NBCn1), and Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter isoform 1 (NKCC1). An additional electrogenic Na\(^+\)-HCO\(_3^-\) cotransporter (NBC4 or NBCe2) is also expressed in heart [27, 28], but its levels of expression are very low. These ion transporters and some aspects of their functional properties will be discussed in the following sections.
The NHE1 Na\(^+\)/H\(^+\) Exchanger

Mammals have multiple isoforms of the Na\(^+\)/H\(^+\) exchanger (NHE), which are distributed in different tissues; however, NHE1 is expressed in all tissues, including the myocardium. Hence, amongst Na\(^+\)/H\(^+\) exchangers it is considered to be the major housekeeping isoform and the major sarcolemmal Na\(^+\)/H\(^+\) exchanger in heart. In addition, none of the other isoforms (NHE2-9) are expressed at high levels, if at all, in cardiac myocytes [24, 29]. Thus, references to sarcolemmal Na\(^+\)/H\(^+\) exchange in heart are almost invariably concerned with NHE1.

**Figure 1.4.** The predicted topology of NHE1, with 12 transmembrane helices in the N-terminal regions of the protein [24, 29] and a hydrophilic C-terminal domain that is located in the cytosol and contains multiple phosphorylation sites and binding sites for regulatory proteins such as calmodulin.

The protein product of the mammalian NHE1 gene (Slc9a1) is 815 amino acids in length with a molecular weight of 91 kDa. However, due to glycosylation (both O- and N-linked) the apparent molecular weight is approximately 110 kDa. The whole protein can be subdivided into two major domains: first, the N-terminal transmembrane domain which consists of 500 amino acids; second, a highly hydrophilic C-terminal cytoplasmic domain with 315 amino acids [24,
The N-terminal domain is responsible for ion exchange activity, while the C-terminal domain plays an important role in the regulation of NHE1 activity. The latter can be emphasized by the presence of several regulatory protein binding domains as well as phosphorylation-targeted amino acid residues (Fig. 4). The glycosylation of NHE1, and particularly the N-linked glycosylation, is suggested to play a crucial role in NHE1 sorting to the basolateral membrane of polarized epithelial cells. As yet, the crystal structure of NHE1 is not available. However, hydropathy investigations along with protease cleavage analysis predict a topology model (Fig. 1.4) with 12 transmembrane segments [30, 31].

Regulation of NHE1

The concentration of the intracellular hydrogen ions (pHi) is thought to be a major factor in the activation of NHE1. However, the exact mechanism of such activation is not clearly understood. One possibility is that protons bind to a H⁺-sensor (schematized in Fig. 1.4) located on the intracellular face of the exchanger [24, 29], with subsequent structural conformations triggering extrusion of H⁺ with simultaneous influx of Na⁺. In addition, several metabolic pathways have been found to be involved in the activation of NHE1, specifically in the myocardium. For instance, activation of NHE1 can be induced by catecholamines, such as norepinephrine (NE), which mediate induction through binding to the α1 adrenergic receptors (α1-ARs) [32]. In addition, angiotensin II (ANG II) has been found to stimulate NHE1 activity during ischemia/reperfusion (I/R) either directly or indirectly through endothelin-1 (ET1) [33]. Moreover, the MAPKs, namely extracellular-signal regulatory kinases (ERK1/2), are implicated in NHE1 stimulation after hypoxia and ischemia/reperfusion. This stimulation occurs either directly, or through p90 ribosomal S kinase (p90RSK) as the downstream effector protein [34,
In line with these findings, it has recently been shown that inhibition of p90RSK gives rise to protective effects in response to I/R injury in vivo [36]. In addition, NHE1 is activated by protein kinase C (PKC) in a downstream cascade involving phosphoinositide (PI) hydrolysis via phospholipase C (PLC). NHE1 stimulation can also be induced in vitro by mechanical stretch of cardiomyocytes, with subsequent activation of Raf1 and MAPK that results, in turn, in cardiomyocyte hypertrophy [37]. Finally, a number of ischemia-associated compounds, e.g. lysophosphatidylcholine and H₂O₂, have been found to activate NHE1 [38]. Thus, if some of the AE3-mediated Cl⁻/HCO₃⁻ exchange in heart were coupled with NHE1, thereby facilitating an increase in Na⁺ influx, it could have a major effect on processes affected by NHE1.

**The NKCC1 Na⁺-K⁺-2Cl Cotransporter**

NKCC1 is expressed in almost all tissues, with highest levels in epithelial tissues, where it plays a major role in transepithelial secretion of Cl⁻ [39, 40]. In contrast, a second Na⁺-K⁺-2Cl cotransporter isoform, NKCC2, is expressed only in the kidney [24]. Both isoforms utilize the inwardly directed Na⁺ and Cl⁻ gradients to mediate electroneutral transport of 1 Na⁺, 1 K⁺, and 2 Cl⁻ into the cell. Although NKCC1 is often viewed as functioning primarily in epithelial tissues, phenotypic analyses of NKCC1 null mutant mice, discussed in a later section, show that it has important functions in cardiovascular tissues as well.

NKCC1 is approximately 1200 amino acids in length and has a molecular weight of about 130 kDa (without glycosylation) [24, 25]. Again, for NKCC1 a high-resolution crystal structure is not yet available; however, hydropathy analysis showed a topology of 12 membrane-spanning domains with N-linked glycosylation that is associated with the fourth extracellular loop (Fig. 1.5). The general structure of NKCC1 revealed three distinct regions: a central hydrophobic region (encompassing the 12 transmembrane segments) (about 50 kDa), and two
flanking N-terminal (~20-30 kDa) and C-terminal (~50 kDa) regions that are located in the cytosol. A number of regulatory protein binding domains have been identified in the N-terminal region. In addition, different phosphorylation consensus sites have been recognized both in the N-terminal and C-terminal regions. For instance, three threonine residues in the N-terminal cytosolic domain were found to be phosphorylated in different species [24, 25].

Figure 1.5. A proposed topology model for the NKCC1 cotransporter [25] showing extensive cytosolic domains at both the N-terminus and C-terminus and a central membrane embedded domain with at least 12 transmembrane helices.

Regulation of NKCC1.

Previous studies indicate that phosphorylation of NKCC1 can lead to its activation [25], and sequence analysis suggested that 20 consensus amino acids might be involved in such phosphorylation-associated processes. In rat myocardium, NKCC1 was shown to be phosphorylated and activated by catecholamines acting on α1-adrenergic receptors in an ERK1/2-dependent pathway [41]. In addition, angiotensin II was suggested to induce NKCC1 activity in cardiac myocytes [41]. More recently, a macromolecular complex with
phosphorylation activity has been shown to be involved in the regulation of NKCC1. This complex includes members of novel serine-threonine families, namely, the WNK kinases and the Ste-20-related kinases SPAK and OSR1 [42, 43]. It has been found that Wnk3 kinase colocalizes with NKCC1 and that activation of this kinase is associated with an increase in Cl⁻ influx [44].

**The NBCe1, NBCe2, and NBCn1 Na⁺/HCO₃⁻ Cotransporters**

The cardiac variant of the electrogenic Na⁺/HCO₃⁻ co-transporter (hhNBC; NBCe1; encoded by *Slc4a4*) was first cloned from a human heart cDNA library and found to comprise 1035 amino acids [45]. This variant had an identical amino acid sequence to that of the human kidney NBCe1 (hkNBC) with the exception of the first 41 amino acids in kidney NBCe1. These 41 amino acids were replaced with 85 amino acids in hhNBC. The alternative 85-amino acid sequence in hhNBC contained a higher percentage of charged amino acids residues (50%) than the 41-amino acid sequence in hkNBC (22%). The difference between hhNBC and hkNBC is due to the use of alternative promoters from the same gene. Moreover, another variant has been described from pancreatic tissue (pNBC) that is 100% identical in amino acid sequence to the hhNBC isoform; however, the 5'-UTR region of the pancreas and heart mRNAs are quite different [45, 46]. The difference is likely to be due to the use of tissue-specific promoters, thus leading to alternative 5'-UTRs. Structural studies of the genes involved revealed that all of the homologous isoforms (hNBC, kNBC and pNBC) are transcribed from the same gene [47]. All isoforms have a regulatory PKA binding site in the carboxy terminal side, however, the extra 85 amino acids (N-terminal side) in hhNBC (and pNBC) also have a putative PKA binding site [45]. It is believed that differential regulation of NBCe1 in cardiac myocyte (and pancreatic tissues) is mediated through the N-terminal PKA binding site [45].
In addition to NBCe1, a second electrogenic NBC isoform, termed NBC4 or NBCe2, is expressed in heart. First identified in human heart [27], it was reported as being absent in rat heart. Unpublished Northern blot analyses by our laboratory indicate that NBC4 (NBCe2) is expressed in mouse heart, however, its levels are very low and it is unclear whether it plays a significant role in heart. It should be noted that the nomenclature for the NBC isoforms has been a major source of confusion, with different investigators sometimes using the same symbol when referring to different NBCs. The designation NBCe1 and NBCe2 to specify the two electrogenic NBC isoforms and NBCn1 and NBCn2 (which is not in heart and was not discussed) now being commonly used to designate the two electroneutral NBC isoforms.

Another important member of the NBC family is NBCn1 (previously termed NBC2 or NBC3) that mediates the electroneutral cotransport of \(1\text{Na}^+\) and \(1\text{HCO}_3^-\). NBCn1 is encoded by the \(\text{Slc4a7}\) gene and comprises 1218 amino acids, with a broad tissue distribution including heart, kidney and skeletal muscle [5]. Analysis of NBC isoforms in heart tissue from human patients with ischemic and dilated cardiomyopathy show that NBCe1, but not NBCn1, is increased [48]. However, a recent study showed that both NBCn1 and NBCe1 are upregulated in hearts of rats subjected to chronic pressure overload hypertrophy by aortic constriction [49].

All \(\text{Na}^+/\text{HCO}_3^-\) cotransporters are involved in intracellular pH regulation, either through an inward or an outward (which appears to occur only in the renal proximal tubule) mode of action. NBCs in the cardiac myocyte function as acid extruders (\(\text{Na}^+\) and \(\text{HCO}_3^-\) influx) and it has been suggested that, under physiological conditions, both NBC and NHE1 activities may be equally important in acid extrusion, with the balance shifted toward NHE1 under pathological conditions [2]. Overall, increased NBC activity would be expected to increase \([\text{Na}^+]_i\), with a subsequent increase in \([\text{Ca}^{2+}]_i\). If AE3-mediated Cl\(^-\)/\(\text{HCO}_3^-\) exchange is coupled with a \(\text{Na}^+-\)
dependent acid extrusion mechanism to facilitate Na\(^+\)-loading, either NBCn1, NBCe1, or NHE1 would be reasonable candidates.

**NHE1 and Cardiac Ischemia/Reperfusion Injury**

The major role of NHE1 in I/R injury can be appreciated through the exchanger-coupled theory [50]. This coupling theory entails that shortage of blood flow in the affected tissues (in this case the myocardium) induces an anaerobic pathway for energy production, i.e., through glycolysis. This in turn will lead to the production of lactic acid and accumulation of hydrogen ions with subsequent acidosis of the intracellular milieu of the cell. Hence NHE1 will be activated with a robust extrusion of surplus protons in exchange for sodium ions. The subsequent accumulation of intracellular Na\(^+\) leads to reversal or inhibition of the NCX1 Na\(^+\)/Ca\(^{2+}\) exchanger, which in turn leads to accumulation of intracellular Ca\(^{2+}\). Subsequently, this leads to the observed cardiac damage in patients experiencing an ischemic myocardium [51, 52]. A summary of the coupled exchanger theory is schematized in Figure 1.6.

Several mechanisms have been suggested to mediate injury through accumulation of calcium ions, including activation of proteolytic enzymes and disruption of mitochondrial functions [51]. The central role of NHE1 in this damaging process has been shown by numerous animal studies, either by using therapeutic inhibition of NHE1 [53, 54] or through genetic ablation of the exchanger [55]. Inhibition or loss of NHE1 activity in these studies leads to amelioration of the I/R-related cell injuries. Indeed, this was one of the rationales on which several clinical trials have been initiated to investigate the clinical benefits of NHE1 inhibition in patients at high risk for I/R injuries [56, 57].
NHE1 and Cardiac Hypertrophy

Cardiac hypertrophy represents an early indicator of subsequent morbidity in the myocardium that will result ultimately in heart failure (the inability of the heart to fulfill its pumping function). In a simplified scheme of hypertrophy, the myocardium, in order to cope with the increased pumping needs, will increase muscle mass and consequently increase the overall size of the heart.

Figure 1.6. The coupled exchanger theory in I/R injury: 1) in ischemia, anaerobic glycolysis will lead to accumulation of $H^+$ and lactate ($L^-$), 2) intracellular acidosis will activate NHE1 resulting in elevation of intracellular sodium ions $[Na^+]_i$, 3) increased $[Na^+]_i$ leads to inhibition or inactivation of the forward mode of NCX or to activation of the reverse mode, with increases in $[Ca^{2+}]_i$ [51]. Note: the scheme in this figure shows NCX in the reverse mode.

The progression to cardiac hypertrophy, specifically compensatory hypertrophy, and subsequent heart failure are inherently correlated with a disturbance of $Ca^{2+}$ handling. This is reflected by an increase in diastolic $Ca^{2+}$ levels and to a decrease in sarcoplasmic reticulum (SR) $Ca^{2+}$ content [58, 59]. In other words the increase in free intracellular $Ca^{2+}$, mainly via inhibition of the forward mode of NCX1 or activation of the reverse mode, leads to diastolic dysfunction.
However, several other Ca\textsuperscript{2+} handling proteins, in addition to NCX1, are involved in the Ca\textsuperscript{2+} disturbance, including SERCA2 (the SR Ca-ATPase) and the ryanodine receptor (RyR) which functions as the SR Ca\textsuperscript{2+} release channel [60-63].

In addition to calcium, both cardiac hypertrophy and heart failure are correlated with an increase in intracellular sodium ions [Na\textsuperscript{+}]. This increase in [Na\textsuperscript{+}], is attributed to either of two factors: a decrease in Na\textsuperscript{+} efflux, in which the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase serves as the main mechanism for Na\textsuperscript{+} efflux, or an increase in Na\textsuperscript{+} influx, which is mediated via a number of Na\textsuperscript{+} transporters. Some studies support the first mechanism, i.e., a decrease in Na\textsuperscript{+} efflux mediated via a decrease in Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity [63]. However, other studies showed that such an increase in [Na\textsuperscript{+}], is mediated through NHE1 [64], i.e., by the second mechanism through an increase in Na\textsuperscript{+} influx.

The increase in [Na\textsuperscript{+}], will lead ultimately to an elevation of intracellular calcium ions through the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. This mechanism is mediated through the coupling interactions between NHE1 and NCX1 as mentioned above. It is interesting to note that higher pH\textsubscript{i} (through H\textsuperscript{+} extrusion via the hyperactive NHE1), along with high [Ca\textsuperscript{2+}],, are both associated with an increase in protein synthesis [52]. The increase in protein synthesis at higher pH\textsubscript{i} values is estimated to be ~40% with respect to an increase of 0.1 units in pH\textsubscript{i} [52]. In addition, other investigators have investigated the role of NHE1 activity in cardiac hypertrophy from a different perspective, in which the inhibition of NHE1 leads to a reduction in cardiac hypertrophy and heart failure [65, 66]. Moreover, Baartscheer et al. [67] showed that chronic inhibition of NHE1 by cariporide, which is highly specific for NHE1, leads to attenuation of cardiac hypertrophy in rabbit hearts and to the inhibition of heart failure. They attributed the therapeutic effect of inhibiting NHE1 on heart failure to several possible mechanisms: first, a role mediated through inhibition of protein kinase C (PKC) (and its signaling pathways) that has been found to be
associated with high Na$^+$ influx [68], and second, by interrupting the increase in [Ca$^{2+}$]$_i$ that occurs through the coupling of NHE1 and NCX1 activities. Under normal conditions, an elevation of [Ca$^{2+}$]$_i$ activates MAPKs and RAF-1 kinase, with subsequent induction of cell growth [69]. In addition, inhibition of NHE1 should prevent the increase in protein synthesis that is stimulated by elevated pHi, as mentioned above.

**Analysis of Cardiac Functions of NHE1 and NKCC1 using Knockout Mice**

The use of gene-targeting technology, in which a specific gene can be disrupted or modified is an extremely valuable means of analyzing the biological functions of encoded proteins. Among the transporters that might interact directly with AE3 or carry out a similar function in heart, null mutant mouse models have been prepared for NHE1 and NKCC1. The phenotypes of those models and their similarities or dissimilarities with the phenotype of AE3 null mice are discussed below.

**NHE1-Null Phenotype**

Because NHE1 is expressed in most if not all tissues, it was expected that its absence would cause a severe phenotype in null mutants. NHE1 is considered to be a major housekeeping transporter for regulation of pHi and cell volume, and also serves a number of tissue-specific functions, so early embryo lethality was expected. However, the knockout animals showed normal mendelian ratios at birth, suggesting that its absence caused no major disturbance of embryogenesis [70]. Differences in the phenotypes of wild-type and NHE1-null mice become apparent by the 2nd week after birth, with the occurrence of growth retardation, which persists throughout adult life. For instance, at 10 weeks of age null mutants have an average body weight that is only 60-70% of wild-type body weight [70]. The animals show a high rate of early death,
which can be as high as 70% before weaning. During the second week, null mutants display an ataxic gait, “hesitant behavior”, and hyper-excitability [69]. Much of the behavioral phenotype is attributable to neurological functions of NHE1 [71]. Mutant mice exhibit distinct neurodegeneration in cerebellar, vestibular and cochlear nuclei, and develop epilepsy [71].

The effects of NHE1 ablation on ischemia-reperfusion (I/R) injury was analyzed using the isolated Langendorff heart preparation. The protective effect of NHE1 ablation was robust with less impairment of NHE1-null hearts in comparison with wild-type hearts [55]. In addition, pharmacological inhibition of NHE1 in the same study revealed similar protective effects in WT hearts treated with the NHE1 inhibitor eniporide and no additional protective effects on null mutant hearts. These results confirm earlier reports, although with a genetic rather than a pharmacological approach, of the beneficial effects of NHE1 inhibition [65]. Most likely, these protective effects are related to the established role of NHE1 in Na⁺ loading and its direct effects on NCX1 activity. Hence, inhibition or genetic ablation of NHE1 leads to inhibition of NCX1 activity with a subsequent reduction in [Ca²⁺]ᵢ and prevention of the Ca²⁺-induced hypercontracture injury.

Similar studies using Langendorff ex vivo heart preparations were conducted to determine the effects of I/R injury on AE3-null hearts [1]. In contrast to loss of NHE1, the loss of AE3 had no apparent effect on the degree of I/R injury. These results suggest that if NHE1 operates in concert with AE3 to facilitate Na⁺-loading under certain conditions, it is unlikely that such coupling occurs during ischemia and reperfusion. Whether there are other circumstances in which AE3 and NHE1 might operate together is yet to be determined.
**NKCC1-Null Phenotype**

In an attempt to define the physiological functions of NKCC1, Flagella et al. disrupted the gene encoding NKCC1 in mice (locus *Slc12a2*) [39]. Genotype analysis showed the normal Mendelian birth ratio of 1:2:1, corresponding to homozygous wild-type, heterozygous, and homozygous mutants, respectively. This provides evidence that NKCC1 disruption does not affect normal embryonic development. However, the knockout animals showed growth retardation by the third week after birth. In addition, roughly 30% of the animals died at around weaning (21 days) and investigation of the dead animals showed evidence of intestinal bleeding as well as blockage of the colon in some animals. Also the knockout animals showed significant reductions in mean arterial pressure as well as reduced Cl⁻ secretion in intestinal tissues when compared with wild-type littermates. Interestingly, live null mutants exhibited circling behavior with a tendency to spin in place that persists throughout their life. Moreover, auditory studies showed that these animals are profoundly deaf, with gross histological abnormalities of the inner ear related to the failure of K⁺ secretion into the endolymph. The authors attribute both the deafness and circling behavior to these abnormalities in the inner ear [39]. In another study involving acinar cells from parotid glands of NKCC1-null mice, Evans et al. showed a dramatic reduction (about 60%) in fluid secretion (saliva) after stimulation with secretagogues [72]. The authors concluded that perturbations of secretion via this mechanism might contribute to the pathophysiology of idiopathic dry mouth defects in human [72]. Other work in our laboratory has shown that anion secretion in colon is supported by both NKCC1 and the coupled activities of AE2 and NHE1 on basolateral membranes and that loss of either AE2 or NHE1 activity leads to immediate compensation by NKCC1 [40].
Analyses of the NKCC1-null mouse showed that NKCC1 plays a major role in maintaining blood pressure and that loss of its activity affects contractility of vascular smooth muscle [73]. However, other parameters of cardiovascular function, including contractility (+dP/dt) and relaxation (-dP/dt) showed no differences between mutant and wild-type mice. Studies of AE3-null mutants gave similar results, with no apparent effect on contractility or relaxation. In contrast, the combined loss of NKCC1 and AE3 caused a reduction in cardiac contractility, and loss of either transporter separately caused similar biochemical changes in heart [1]. These observations are consistent with the view that NKCC1 acting alone or AE3 acting in concert with a Na⁺-dependent acid-loading mechanism serves as a means of Na⁺-loading, with accompanying Cl⁻-loading, in heart. The data also suggest that the AE3 can compensate for the loss of NKCC1 in heart and that NKCC1 can compensate for the loss of AE3. These observations suggest complementarity between the functions of NKCC1 and AE3 in heart that might be related to effects on intracellular Na⁺ homeostasis.

The Heart and its Diseases

The heart is a pump organ responsible for pumping blood to all parts of the body, thereby providing oxygen and nutrients to every single cell. The cardiac cycle entails two consecutive events: diastole (relaxation) and systole (contraction). Diastole leads to filling of the ventricular chambers with blood whereas systole evacuates the blood, with about two-thirds of the blood being ejected in each contraction. Several interlinked systems of neurohumoral, electrical and mechanical entities are involved in accomplishing this life-sustaining cycle.

During the relaxation phase of the cycle, blood coming from the atrial chambers enters the ventricles with minimum resistance. However, during the contraction phase the ventricles must push blood against an existing resistance. For the right ventricle, in which the blood will be
ejected into the pulmonary circulation for gaseous exchange, the resistance is relatively low (pressure in the pulmonary artery is ~40 mm Hg). In contrast, the left ventricle forces blood into the systemic circulation against a blood pressure of 110-120 mm Hg in the aorta. Hence, the mass and wall thickness of the left ventricle is much larger than that of the right ventricle, reflecting this physiological need. These physiological and anatomical differences between the left and right ventricles are also reflected in disease conditions, with enlargement and remodeling affecting the left ventricle more than the right ventricle.

Heart disease is the primary cause of morbidity and mortality in the Western hemisphere. However, heart disease is attributed to a multitude of etiologies ranging from myocarditis (viral infection in the heart) to valvular heart disease and all the way to genetic cardiomyopathies and coronary heart disease with diverse phenotypic manifestations. In contrast, heart failure (HF) represents an advanced stage of heart disease in which the organ cannot efficiently fulfill its principal function as a pump. On the clinical level, heart failure is classified broadly into four overlapping stages, ranging from Stage-A, encompassing high-risk patients without symptomatic and structural cardiac changes, and up to Stage-D, featuring overt heart failure symptoms that are refractory to standard therapeutic protocols [74] (Table 1.3).
Table 1.3. Classification of clinical severity in heart failure according to established guidelines (reproduced from Ref [75] with original references herein).

<table>
<thead>
<tr>
<th>NYHA Functional Classification</th>
<th>ACC–AHA Stages of Heart Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I No limitation of physical activity; ordinary physical activity does not cause undue fatigue, palpitation, or dyspnea</td>
<td>Stage A At high risk for heart failure; no identified structural or functional abnormality; no signs or symptoms</td>
</tr>
<tr>
<td>Class II Slight limitation of physical activity; comfortable at rest, but ordinary physical activity results in fatigue, palpitation, or dyspnea</td>
<td>Stage B Developed structural heart disease that is strongly associated with the development of heart failure but without signs or symptoms</td>
</tr>
<tr>
<td>Class III Marked limitation of physical activity; comfortable at rest, but less than ordinary activity results in fatigue, palpitation, or dyspnea</td>
<td>Stage C Symptomatic heart failure associated with underlying structural heart disease</td>
</tr>
<tr>
<td>Class IV Unable to carry on any physical activity without discomfort; symptoms present at rest; if any physical activity is undertaken, discomfort is increased</td>
<td>Stage D Advanced structural heart disease and marked symptoms of heart failure at rest despite maximal medical therapy</td>
</tr>
</tbody>
</table>

In the US, more than 5 million patients are diagnosed with heart failure giving rise to an incidence of greater than 1% of the population, and accounting for ~20% of hospital admissions in the ≥ 65 year-old age-group [76]. In addition, mortality from heart failure surpasses all major cancer subtypes giving rise to an annual mortality of 45% in patients having symptomatic heart failure [76]. These statistics give an alarming snapshot of the magnitude of the health problems associated with cardiac disease and failed hearts.

One of the major categorical etiologies leading to heart disease/failure at an early age is cardiomyopathies attributed mainly to genetic alterations in one or more of the sarcomere component proteins (sarcomere is the basic contractile apparatus in cardiomyocyte) (Fig. 1.7). The majority of patients having this sort of heart disease harbor an underlying “private” mutation which is unique to the patient’s own family [77]. However, cardiomyopathies are broadly divided into two major subtypes: hypertrophic cardiomyopathy (HCM) with distinctive enlargement of the myocardium and essentially a defective relaxation (diastole). The other type is dilated cardiomyopathy (DCM), featuring an increase in ventricular volume (dilatation) and compromised contractile function [78]. For the sake of the research in this dissertation, two
different mutations (and the relevant mouse models) will be described. Both of these mutations affect the sarcomeric protein α–tropomyosin and are designated as α–Tm180 and α–Tm54. They affect codon 180 and codon 54, respectively, of the α–Tm gene in the human population [79, 80]. Transgenic mouse models having each of these mutations were developed and described previously [81, 82]. The animal models and relevant mutations will be described briefly in the following sections.

![Schematic diagram featuring the main sarcomeric protein components](image)

**Figure 1.7.** Schematic diagram featuring the main sarcomeric protein components [78].
**α–Tm 180 (Glu180Gly) Transgenic Mouse Model of HCM**

Alpha-tropomyosin is an essential component of the sarcomeric contractile apparatus in cardiomyocytes conferring stability and scaffolding support to F-actin, the major functional protein in the thin filament (Fig. 1.7). Different mutations were described in the α–Tm gene that affect the human population [83]. One of these mutations is designated as Tm180, affecting amino acid 180 of the α–Tm protein product and causing a Glu-Gly missense alteration [80]. The α–Tm 180 transgenic (Tg) mouse model was generated several years ago [81]. The animals develop progressive cardiac hypertrophy with prominent left atrial enlargement. In addition, they display severely compromised heart function affecting both contractility and relaxation [81]. Histological analysis revealed prominent cardiac fibrosis and cardiomyocyte disarray, all of which are established hallmarks of HCM. Interestingly, analyses of calcium-force relationship in isolated skinned fibers revealed a significant increase in Ca\(^{2+}\) sensitivity in mutant myocytes when compared with wild-type. The TM180 mice died prematurely at ~5 months of age [81]. The TM180 model has been used in this thesis project to identify the consequences of AE3 ablation on heart function in the context of HCM.

**α–Tm 54 (Glu-Lys) Transgenic Mouse Model of DCM**

The second animal model of heart disease/failure to be used in this dissertation research is transgenic mouse model with another mutation in the same α–Tm gene. This model, designated as α–Tm 54, was generated using an α–Tm transgene carrying a mutation at amino acid 54 (Glu-Lys) of the α–tropomyosin protein [82]. This mutation was described originally as the causal mutation of a form of dilated cardiomyopathy (DCM) in the human population [80]. The animal model of α–Tm 54 recapitulates the human disease, giving rise to a dilated
cardiomyopathy and leading to frequent premature death in affected animals by as early as 6 months of age. In contrast to the other model (α−Tm 180), a skinned fiber preparation of this model showed a significant reduction in Ca^{2+} sensitivity, in agreement with previous in vitro studies targeting this mutation in α−Tropomyosin [82]. The TM54 model has been used in this thesis project to identify the consequences of AE3 ablation on heart function in the context of DCM. Interestingly, the results indicate that loss of AE3 leads to different effects on Ca^{2+} handling in the two models, which differ in Ca^{2+} sensitivity of the myofibrillar apparatus.

**Calcium Cycling in Cardiomyocytes**

Calcium is the primary second messenger in cardiomyocytes. It plays a central role in regulating contraction of the myofilaments, and hence the beating of the whole heart. Changes in contraction involving alterations in Ca^{2+} can occur by at least two possible routes: changes in the Ca^{2+} transient amplitude and its decay time or duration, and changes in myofilament Ca^{2+}-sensitivity, i.e., the relative amount of Ca^{2+} needed to activate the myofilaments [84]. Overall, to accomplish its function in muscle contraction and relaxation, Ca^{2+} handling follows a cycle in cardiac myocytes, beginning with excitation, which leads to increased cytosolic Ca^{2+} coupled with contraction and ends with relaxation coupled with the removal of Ca^{2+} from the intracellular milieu by sequestration in the sarcoplasmic reticulum (SR) or extrusion from the cell. Ca^{2+} influx and Ca^{2+} efflux must be tightly coordinated so the cells will not lose or gain Ca^{2+} over time.

**Excitation and Contraction:** During the initial excitation phase, changes in membrane action potential, namely a depolarization event, trigger the instantaneous gating of voltage-activated Ca^{2+} channels (L-type Ca^{2+} channels, LTCCs; or dihydropyridine receptor, DHPR). The opening of LTCCs leads to an influx of Ca^{2+} from the extracellular side to the intracellular
side of the sarcolemma. Due to the rapid inactivation of LTCC, due to the increased [Ca\(^{2+}\)], this small influx of Ca\(^{2+}\) is not sufficient per se to efficiently activate myofilament contraction. Hence, the principal role of this small level of Ca\(^{2+}\) influx is to activate and trigger the release of large amounts of Ca\(^{2+}\) from SR Ca\(^{2+}\) stores by activating the ryanodine receptors (RyR2, the SR calcium-release channel), in a process known as Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) [84]. SR Ca\(^{2+}\) stores can be increased by increasing Ca\(^{2+}\) influx via LTCC, decreasing Ca\(^{2+}\) efflux via NCX1, or increasing SR Ca\(^{2+}\) uptake via the SERCA2 Ca\(^{2+}\) pump.

The surge of Ca\(^{2+}\) from the SR leads to activation of myofilament contraction mediated by Ca\(^{2+}\) binding to the cardiac troponin C subunit (cTnC) of the troponin complex. Specifically, Ca\(^{2+}\) binding triggers conformational changes involving the inhibitory TnI subunit and α–tropomyosin. This leads to unmasking of the myosin-interacting sites on the actin filament. Finally, the interaction between myosin heads (thick filaments) and actin (thin filaments) initiates the actomyosin reaction leading to the cross-bridge sliding of thick and thin filaments against each other, resulting in the observed contraction [85].

**Calcium removal and relaxation:** The last stage of the cycle is removal of Ca\(^{2+}\) from the myofilaments and intracellular milieu. This occurs by Ca\(^{2+}\) uptake into the SR via the SR calcium pump (SERCA2) and Ca\(^{2+}\) efflux from the cell via the NCX1 Na\(^{+}\)/Ca\(^{2+}\) exchanger. Phosphorylation of TnI at Ser-23/24, mediated by PKA, is a well-established mechanism for desensitizing the myofilament toward Ca\(^{2+}\) and enhancing the release and cycling of Ca\(^{2+}\). In addition, phospholamban (PLN) in the SR functions as an intrinsic regulator of SERCA2. When PLN is phosphorylated at Ser16, again via PKA, the inhibitory effect of PLN on SERCA2 will be relieved, enhancing the sequestration of Ca\(^{2+}\) into the SR. PKA activation is the major route for enhancing contractility and Ca\(^{2+}\) cycling in cardiomyocytes downstream of β–adrenergic
receptor activation. Figure 1.8 summarizes the steps involved in excitation-contraction coupling and the experimental recordings from isolated rabbit cardiomyocytes [84].

Figure 1.8. Schematic diagram summarizing the processes involved in excitation-contraction (EC)-coupling and the subsequent removal of calcium. The inset shows recordings of electrical excitation, followed by release of calcium, and finally contraction from rabbit ventricular cardiomyocytes (adapted from Ref.[84] ).

Research Objectives

My objective in this thesis is to decipher the role of AE3 ablation in diseased heart conditions. Ramifications of AE3 ablation will be analyzed on all levels (whole animal, cellular and molecular) by using three well-established mouse models of heart disease:

1. Transgenic mouse model of Tm180, a model of hypertrophic cardiomyopathy (HCM).

2. Transgenic mouse model of Tm54, a model of dilated cardiomyopathy (DCM).

3. Angiotensin II infusion via implanted minipumps, a model of pressure overload heart disease.
Chapter 2: Materials and Methods

Generation of mutant mice

Gene-targeted AE3 heterozygous mutant (AE3 +/-) mice [1] and transgenic mice carrying a Glu180Gly mutation (TM180) or a Glu54Lys (TM54) in α-tropomyosin [81,82], both on an inbred FVB/N genetic background, were used for the generation of mice for these experiments. Mice were kept under 12 h dark-light cycles and fed regular chow with temperature and humidity maintained as recommended. All procedures conformed to guidelines published by the National Institutes of Health (Guide for the Care and Use of Laboratory Animals; Publication No. 86-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Cincinnati.

Measurement of Heart and lung weight to Body Weight Ratio

Heart to body weight ratios (HW/BW) were obtained by measuring the whole heart weight (in mg) after brief rinsing in PBS and trimming of extra tissues and dividing it by the body weight (in g). For calculation of the lung weight to body weight ratio we measured only the left lobe of the lung (a single lobe) and divided its weight (in mg) by the body weight (in g).

Measurement of Fluid in the Chest Cavity

Measurement of thoracic fluid was obtained by carefully opening the chest cavity via a cut through the diaphragm to avoid massive bleeding and spillage of the fluid. The volume of accumulated fluid was measured by using a micropipettor.
Evaluation of Cardiac Function in vivo using the closed chest model

Analysis of cardiovascular function was performed as described previously [86, 87]. All of these experiments, as well as the force-frequency experiment (see below), were conducted in the laboratory of Dr John Lorenz of the Department of Cellular and Molecular Physiology. Briefly, the mice were anesthetized with a mixture of Ketamine (50 μg/g BW) and Inactin (100 μg/g BW) and their body temperatures were monitored with a rectal probe and maintained constantly at 37 °C by using a thermally controlled surgical stage. The right femoral artery was cannulated with a catheter that was connected directly to a pressure transducer for measurements of mean arterial pressure. In addition, the right femoral vein was cannulated with a catheter for delivery of drugs, including a series of increasing doses of Dobutamine, via a microliter syringe that was mounted in a microinjection pump. A high fidelity pressure transducer (Millar Instruments, Houston, TX) was advanced into the left ventricle (LV) via the right carotid artery and ascending aorta and used to analyze left ventricular function. A PowerLab data-acquisition system (AD Instruments, Colorado Springs, CO) connected to a Macintosh computer was used for data analysis and derivation of the different indices. The schematic diagram in Figure 2.1 illustrates the overall procedures for in vivo analysis of heart function using these procedures.

Force frequency experiment

Force frequency analysis was accomplished essentially as described before [17]. Briefly, mice were anesthetized with a mixture of Ketamine and Inactine, as described above, and heart
rates were electrically paced via an angioplasty guide wire that was placed on the right atrium. Increments in heart rate were adjusted by a gradual increase in the frequency of pacing (in Hz).

**Figure 2.1.** Schematic diagram illustrating the procedure used for the closed-chest in vivo evaluation of heart function in mice.
Immunoblot analyses

Mice were anesthetized with 2.5% Avertin (15 µl/g bodyweight) and allowed to stabilize for 10 minutes on a thermally controlled heating pad. Ventricles were collected, rinsed briefly in chilled 1X PBS, snap-frozen in liquid nitrogen, and stored at -80 ºC for future processing. In studies designed to analyze phosphorylation of phospholamban (PLN) in response to β–adrenergic stimulation, ventricles were collected from mice that had been anesthetized with ketamine and inactin and surgically instrumented as described in the previous section. Intravenous infusion of Dobutamine (32 ng/g/min) was performed for 5 min before collection of ventricles. Ventricular homogenates were prepared in 10 mM Tris-HCl (pH 7.4) buffer containing 1 mM EDTA, 2 mM DTT, 0.25% Nonidet P-40, 0.5% Triton X-100, and both protease and phosphatase inhibitors (Catalog numbers P8340 and P5726, Sigma, USA). Homogenization was accomplished on ice using a Brinkmann Polytron mechanical homogenizer (20s, 7000 rpm and 30s, 15000 rpm). Extraction of myofibrillar fractions was performed as reported before [1]. Briefly, 3-4 mg of cardiac homogenate was diluted to 1 ml with 20 mM Tris (pH 7.4) buffer containing protease and phosphatase inhibitors and homogenized on ice for 1 min at 13000 rpm, followed by centrifugation for 10 min at 3000 rpm at 4 ºC. Finally, the cytosolic fraction was removed and the myofibrillar fraction was dissolved in the same homogenizing buffer mentioned above. Protein was estimated using a modified Bradford assay (Thermo Scientific), separated by SDS-PAGE, transferred onto nitrocellulose or PVDF membranes, incubated with primary antibodies followed by the corresponding secondary antibodies, and protein signals were obtained using the KPL Lumi-Glochemiluminescent substrate system (KPL, Gaithersburg MD, USA). The overall Western blotting procedures are
schematized in Figure 2.2. The primary antibodies for SERCA2a (Catalog number ab2861, abcam, USA), PLN (Catalog number MA3-922, ABR, USA), phospho-Ser16 and phospho-Thr17 of PLN (Catalog Numbers A010-12 and A010-13, Badrilla, UK), the NCX1 Na\(^+\)/Ca\(^{2+}\) exchanger (Catalog number R3F1, Swant, Switzerland), protein phosphatase 1 catalytic subunit (PP1) (Catalog number MAB3000, R&D, USA), and the non-methylated form of protein phosphatase 2A catalytic subunit (PP2A) (Catalog number 4957, Cell Signaling, USA) were used as published before [1]. Analysis of total PP2A after cold-base treatment to demethylate the C-terminus was as described before [1]. Briefly, after protein transfer, the membranes were treated with ice-cold 0.2 N NaOH with gentle shaking for 5 minutes, followed by brief rinsing for 3-4 times in cold TBST (Tris buffered saline + Tween-20) and two washes for 5 minutes each in cold TBST. At this stage the membranes were ready for blocking and other steps employed for Western blotting (Fig. 2.2). Other antibodies were also used including: an antibody against both the full-length and cardiac variants of AE3 [2], NHE1 (Catalog number MAB3140, Chemicon International, USA), L-type Ca\(^{2+}\) channel \(\alpha 2\) subunit (Catalog number MA3-921, ABR, USA), calmodulin-dependent kinase II (CamKII) (a pan-CamKII antibody that recognizes \(\delta\) and other isoforms; Catalog number 611292; BD Transduction Laboratories, USA), \(\beta\)-MHC (Catalog number M8421, Sigma, USA), ANP (Catalog number sc-20158, Santa Cruz, USA), TnI and TnI-p (Ser 23/24) (Catalog numbers 4002 and 4004, Cell Signaling, USA) and RyR2 (Catalog number MA3-916, Thermo Scientific, USA). Lastly, antibodies against s-actin (Catalog number A2172, Sigma, USA) or GAPDH (Catalog number MAB374, Millipore, USA) were used for loading controls.
A final remark regarding the transfer buffer used is that for all proteins, with the exception of RyR2, we used a regular transfer buffer as follows: 39 mM glycine, 48 mM Tris, 0.037% SDS, 10-20% methanol, adjusted to pH 8.3 with HCl. For RyR2 transfer, SDS was omitted and no HCl was added but the buffer included 192 mM glycine, 25 mM Tris, and 20% methanol. Another exceptional condition for analysis of RyR2 was that protein samples (in loading buffer) were incubated at 55 °C for ~1h before loading onto SDS-PAGE. Our standard transfer current is 390-400 mA for ~4h at 4 °C, with the exception of PLN (and p-PLN) in which the transfer duration was delimited to 1.5-2 h.
Figure 2.2. The overall procedure for Western blotting used in this thesis work.

Preparation of cardiomyocytes

Ventricular cardiac myocytes were prepared from adult mouse hearts (3–4 months old) mounted in a Langendorff apparatus as described previously [88]. Briefly, mice were anesthetized with a mixture of ketamine and xylazine (100 µg/ml) (20 µl/g body weight) and
hearts were collected, cannulated via the aorta and perfused for 5 min at 37 °C with a modified Krebs-Henseleit Buffer (Buffer A) containing (in mM): NaCl 113, KCl 4.7, KH2PO4 0.6, MgSO4·7H2O 1.2, NaHCO3 10, HEPES 10, taurine 30, 2,3-butanedione monoxime (BDM) 10 and glucose 5.5 (pH 7.46). The perfusion buffer was then switched to a digestion buffer that contained in addition to Buffer A components: Liberase Blendzyme IV (or Liberase DL) (Roche) at 0.25 mg/mL, trypsin (Invitrogen) at 0.14 mg/mL and CaCl2 at 12.5 μM final concentration (Buffer B). The enzyme solution was re-circulated as needed until completion of the digestion process. After 10-15 min of perfusion with enzyme solution (Buffer B), hearts became swollen and flaccid and ventricles were dissected and teased with fine forceps and dispersed gently by pipetting through a 1-ml plastic pipette in fresh 2.5 ml Buffer B. Isolated cardiomyocytes were sieved to remove cell debris, and cell suspension solution was brought to 5 ml with Myocyte Stop solution I (MS I) [Buffer A + 10 % bovine calf serum (BCS) + 12.5 μM CaCl2]. Myocytes were allowed to settle in the tube by gravity for 15 minutes and the supernatant was removed. Subsequently, myocytes were resuspended in Myocyte Stop solution II (MS II) [Buffer A + 5 % BCS + 12.5 μM CaCl2], and Ca2+ was gradually introduced up to a final concentration of 1.2 mM CaCl2. Figure 2.3 schematically depicts the procedure used for isolation of cardiomyocytes and loading of Fura2-AM.
Calcium studies

Cells were loaded with Fura2-AM (Molecular Probes) to a final concentration of 2 μM in 2 ml plating medium [MEM medium supplemented with 0.1 mg/ml bovine serum albumin (BSA), 2 mM L-glutamine and 10 mM BDM] for 15 min at room temperature in the dark. Probenecid (Molecular Probes) was included as inhibitor of Fura2-AM efflux in the loading solution at 0.5 mM final concentration. Cells were then washed for 20 min in Buffer A (containing 1.2 mM CaCl₂ but no BDM) and cell pellets allowed to settle in the tube for 10 minutes. Supernatant was removed and cells were resuspended again in a fresh Buffer A (containing 1.2 mM CaCl₂ but no BDM) and used directly for calcium studies.

Fluorescence measurement was accomplished with a dual-beam spectrofluorophotometer (PTI International, Birmingham, NJ) after field stimulation at 0.5 Hz and Ca²⁺ transient amplitudes were obtained by calculating the fluorescence ratio at 340-to-380 nm excitation wave-lengths (R₃₄₀/₃₈₀). Data were acquired using the Felix 3.01 or Felix 32 acquisition software (PTI International) and analyzed by using IonOptix Ionwizard Analysis Software (IonOptix LLC, Milton, MA). For caffeine-induced calcium release experiments the cells were loaded with Fura2-AM as above and stimulated for 30s (0.5 Hz) followed by halt of stimulation for 20s and subsequent addition of 10 mM caffeine solution (final conc.) and measurement of fluorescent signals were accomplished as mentioned above. All calcium imaging experiments were performed at room temperature (21-23 °C).
Figure 2.3. Flow chart showing the procedures used in the isolation of cardiomyocytes and cell loading with Fura2-AM [88].
Cell mechanics analysis

Analysis of cell mechanics was accomplished with cell-edge measurements using a CCD video camera mounted on the reversal microscope (Video edge motion detector, Crescent Electronics) [89]. Briefly, isolated cells (without Fura2-AM loading) were resuspended in Buffer A (containing 1.2 mM CaCl2 but no BDM) and field stimulated at 0.5 Hz frequency. Rod-shaped cardiomyocytes with well-defined longitudinal edges were used for this analysis. Raw data were acquired with Felix 32 acquisition software and exported to Ionwizard analysis software (PTI International) in which FS % and other parameters were obtained.

LTCC Calcium Current Recordings

Electrophysiology and calcium current recording was accomplished by Dr. Ilona Bodi in Cincinnati Children’s Research Laboratories (CCHMC). Briefly, isolated cardiomyocytes were kept at 4 °C in low Cl- and high K+ Kraft-Bruh (KB) solution: L-glutamic acid (50 mM), KCl (40 mM), taurine (20 mM), KH2PO4 (20 mM), MgCl2 (3 mM), glucose (10 mM), EGTA (1 mM), HEPES (10 mM), pH 7.4. Current was recorded with an external solution containing the following (in mM): CaCl2 2, tetraethyl-ammonium chloride (TEA-Cl) 135, 4-aminopyridine (4-AP) 5, glucose 10, HEPES 10, pH 7.3. The internal pipette solution contained the following (in mM): cesium aspartate 100, CsCl 20, MgCl2 1, Mg-ATP 2, Na2-GTP 0.5, EGTA 5, HEPES 5, pH 7.3 with CsOH. Recording of calcium currents was accomplished with the whole-cell patch-clamp configuration. Recordings were obtained with Axopatch 200B amplifier, Digidata 1322A A/D converter, and pClamp 10.01 data acquisition software (Axon Instruments, Union City, CA).
Calcium currents were elicited by depolarizing voltage steps (380 ms) from -40 mV to +70 mV in 10 mV increment from a holding potential of -50 mV at 0.2 Hz. To account for variations in cell size, all mean data were expressed as current density. All calcium current experiments were performed at room temperature.

**Implantation of minipumps**

Mice were anesthetized with 2.5% isoflurane and a small area of the fur was shaved on the left back side of the animal. After local sterilization, a small incision was made and the Alzetminipump (Alzet osmotic minipump model 1004, Durect Co., CA) was implanted subcutaneously. The minipumps, which were loaded with Angiotensin II (Sigma, Cat# A9525) dissolved in normal saline, delivered a constant infusion rate of 1 ng/g/min for 28 days.

**2-D M-mode and Doppler Echocardiography**

Evaluation of heart function by echocardiography was accomplished as described before [90]. Briefly, the mice were anesthetized by inhalation of 2.5% isofluorane and small area of the fur covering the heart was shaved. After placing a small amount of “Echogel” over the heart area the probe was adjusted to obtain Doppler and M-mode parameters. LVEDD and LVESD were measured directly by using the leading edge-to-leading edge convention. FS % was calculated as \( [(\text{LVEDD} - \text{LVESD})/\text{LVEDD}] \times 100 \) and EF % as \( [(\text{LVEDD})^3 - (\text{LVESD})^3]/(\text{LVEDD})^3 \). Parameters were obtained from 3 different heart cycles and averaged.
Statistics

Values are presented as means ± standard error of the mean (SE). Chi-square statistics were used for analysis of Mendelian ratios among animals that were born. Survival analysis was performed using the Kaplan-Meier procedure with statistical significance determined by log-rank analysis. For other procedures, one-way analysis of variance (ANOVA) was used along with two-sided Student's t-test, and a P-value of < 0.05 considered significant.
Chapter 3: Tm180/AE3 Mouse Model

Summary

The AE3 Cl-/HCO3⁻ exchanger is abundantly expressed in the sarcolemma of cardiomyocytes, where it mediates Cl⁻-uptake and HCO3⁻-extrusion. Inhibition of AE3-mediated Cl⁻/HCO3⁻ exchange has been suggested to protect against cardiac hypertrophy; however, other studies indicate that AE3 might be necessary for optimal cardiac function. To test these hypotheses we crossed AE3-null mice, which appear phenotypically normal, with a hypertrophic cardiomyopathy mouse model carrying a Glu180Gly mutation in α-tropomyosin (TM180). Loss of AE3 had no effect on hypertrophy; however, survival of TM180/AE3 double mutants was sharply reduced compared with TM180 single mutants. Analysis of cardiac performance revealed impaired cardiac function in TM180 and TM180/AE3 mutants relative to wild-type mice, with double mutants being more severely affected and exhibiting little response to β-adrenergic stimulation. Increased expression of calmodulin-dependent kinase II and protein phosphatase 1 and differences in methylation and localization of protein phosphatase 2A were observed, but were similar in single and double mutants. Phosphorylation of phospholamban on Ser16 was sharply increased in single and double mutants relative to wild-type hearts under basal conditions, leading to reduced reserve capacity for β-adrenergic stimulation of phospholamban phosphorylation. Imaging analysis of isolated myocytes revealed reductions in amplitude and decay of Ca²⁺ transients in both mutants, with greater reductions in TM180/AE3 double mutants. Thus, in the TM180 cardiomyopathy model, loss of AE3 had no apparent anti-hypertrophic effect but led to further impairment of cardiac performance and Ca²⁺ handling, loss of
β-adrenergic stimulation of contractility and relaxation, and more rapid decompensation and heart failure.

**Introduction**

Anion exchanger isoform 3 (AE3, gene symbol Slc4a3) is one of at least four Cl⁻/HCO₃⁻ exchangers in cardiac myocytes. It consists of two protein variants, a longer full-length form (AE3ₙ) that is expressed in brain and other tissues and a cardiac-specific form (AE3ₜ) that is expressed at very high levels in heart [4, 12, 91]. In addition to AE3, the heart contains moderate levels of both AE1 (Slc4a1) and AE2 (Slc4a2) and high levels of PAT1 (Slc26a6), which can mediate both Cl⁻/HCO₃⁻ and Cl⁻/OH⁻ exchange [92, 93]. Why the heart requires such a high capacity for Cl⁻/HCO₃⁻ exchange is unclear; however, it contributes to regulation of intracellular pH (pHi) [94]. In addition, by operating in concert with Na⁺-dependent acid extrusion mechanisms such as Na⁺/H⁺ exchange or Na⁺-HCO₃⁻ cotransport, it can contribute to pHi-neutral Na⁺ uptake [20, 94].

Inhibition of Cl⁻/HCO₃⁻ exchange in heart has been suggested as a possible cardioprotective strategy in treatment of hypertrophy [95], ischemia-reperfusion (I/R) injury [17, 96], and arrhythmias [4]. There is evidence that AE3-mediated Cl⁻/HCO₃⁻ exchange in cardiac myocytes is increased during hypertrophy and that AE3 operates in concert with the NHE1 Na⁺/H⁺ exchanger [20]. However, because of the lack of specific inhibitors and the abundance and diversity of Cl⁻/HCO₃⁻ exchangers in heart, it is unclear whether AE3 or one or more of the other isoforms in heart is coupled with NHE1. Although NHE1 operating by itself leads to cytosolic alkalinization, which in turn would limit its activity, extrusion of HCO₃⁻ by AE3 and/or other Cl⁻/HCO₃⁻ exchangers would provide a balancing acidification mechanism, potentially
enhancing NHE1 activity and facilitating Na\(^+\)-loading. With both transporters operating together, uptake of Na\(^+\) and Cl\(^-\) would occur with little change in pH\(_i\), and increased subsarcolemmal Na\(^+\) could affect Ca\(^{2+}\) homeostasis via modulation of Na\(^+\)/Ca\(^{2+}\) exchange.

Loss of NHE1 activity is cardioprotective both in hypertrophy and in I/R injury [97], so if loss of AE3-mediated Cl\(^-/\)HCO\(_3^-\) exchange reduces NHE1 activity it could be cardioprotective. On the other hand, in studies of the spontaneously hypertensive rat using an inhibitory antibody directed against AE3, no reduction in hypertrophy was observed [95]. Also, genetic ablation of AE3 in mouse had no protective effect on cardiac I/R injury as observed in the Langendorff heart model [1] and the combined loss of AE3 and the NKCC1 Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, neither of which by itself impaired cardiac performance, led to reduced contractility [1]. Thus, even if inhibition of Cl\(^-/\)HCO\(_3^-\) exchange could serve cardioprotective functions under some conditions, the specific role of AE3-mediated Cl\(^-/\)HCO\(_3^-\) exchange is unclear and it is possible that one or more of the other Cl\(^-/\)HCO\(_3^-\) exchanger isoforms in heart might be responsible.

To better understand the functions and importance of AE3 in the heart and to assess whether loss of its activity might be cardioprotective, we analyzed double mutant mice carrying null mutations in AE3 and harboring a Glu180Gly mutation in \(\alpha\)-tropomyosin (TM180) [81]. Previous studies indicated that loss of AE3 alone had no adverse effects on cardiac performance [1]. The mutant \(\alpha\)-tropomyosin transgenic mice, which serve as a model for a familial hypertrophic cardiomyopathy that occurs in humans [79], develop cardiac hypertrophy and begin dying at about 4 months of age [81]. Although the degree of hypertrophy in TM180 mutants was unaffected by the loss of AE3, double mutants exhibited severe deficits in cardiac performance, \(\beta\)-adrenergic responses, and Ca\(^{2+}\) handling, and they developed heart failure much more rapidly.
than TM180 single mutant mice. These results show that AE3 plays an important role in maintaining cardiac function in the TM180 model of familial hypertrophic cardiomyopathy and that ablation of its activity does not have any apparent cardioprotective effects in this model.

Results

Effects of AE3 ablation on survival and hypertrophy in TM180 mice

AE3 heterozygous (AE3\(^+/-\)) female mice were bred with AE3\(^+/-\) males harboring the TM180 mutation. Offspring of the expected genotypes, including TM180/AE3 double mutants, were born in normal Mendelian ratios, indicating that the mutations caused no increase in embryonic or fetal death rates. When TM180 single mutants and TM180/AE3 double mutants were aged, double mutants appeared sicker than single mutants at 2-3 months of age, as indicated by rough coats, labored breathing, and reduced activity, and males appeared to be more severely affected than females. Survival analysis (Fig. 3.1A) showed a dramatic reduction in survival of TM180/AE3 double mutants when compared with TM180 single mutants. However, hypertrophy, as measured by heart weight/body weight ratios, was the same in both single and double mutants (Fig. 3.1B) and increased expression of the \(\beta\)-myosin heavy chain, a marker of hypertrophy, was the same in both mutants (Fig. 3.1C). Thoracic fluid, which was negligible in wild-type (WT) mice, was significantly greater in TM180/AE3 double mutants than in TM180 single mutants (Fig. 3.1D). Compared with WT controls, left lung weight/body weight ratios were elevated to the same degree in both single and double mutants (Fig. 3.1E).
Figure 3.1. TM180/AE3 mutant mice exhibit reduced survival but no change in hypertrophy compared to TM180 single mutants. (A) Survival curves of transgenic TM180 mice (TG) and TG mice with AE3 knocked out (TG/KO); n = 26 TG and 38 TG/KO male mice; p < 0.0001 by Kaplan-Meier log-rank analysis. (B) Heart weight/body weight ratios for 2.5-month-old male and female wild-type (WT), TG, and TG/KO mice showed similar hypertrophy in TG (6.84 ± 0.02 mg/g) and TG/KO (6.91 ± 0.01) compared with WT (4.97 ± 0.04); n = 20 WT, 22 TG, and 23 TG/KO mice; *p < 0.001 vs WT. (C) Immunoblot analysis showed upregulation of β–myosin heavy chain (β–MHC) in ventricles of 2.5- or 3-month-old TG and TG/KO mice; n = 6 males of each genotype; †p < 0.01 vs WT. (D) Accumulation of thoracic fluid was higher in 2.5-month-old TG/KO than in TG mice; n = 17 TG and 14 TG/KO male and female mice; †p < 0.05 vs TG; thoracic fluid was not observed in WT mice (n=10) (E) Left lung weight/body weight ratios (LLW/BW in mg/g) were similarly elevated in 2.5-month-old male and female TG and TG/KO mice; n = 9 WT, 8 TG and 10 TG/KO; †p < 0.01 vs WT.
Histological Analyses of Tm180 and Tm180/AE3 Mutant Hearts

Analysis of histology sections revealed gross enlargement of the hearts in Tm180 and Tm180/AE3 versus WT sections. Prominent enlargement of the atria, especially the left atrium, was uncovered for both Tm180 and Tm180/AE3 heart sections (Fig. 3.2). The degree of cardiac hypertrophy and remodeling indeed underscores the same observations found originally in Tm180 transgenic mice [81]. A comparable level of fibrosis was also observed in both Tm180 and Tm180/AE3 hearts. However, double mutant hearts seem to display dilation when compared with Tm180 heart sections. Development of dilatation in Tm180/AE3 heart sections is in accordance with the accelerated progression of heart failure and cardiac decompensation in Tm180/AE3 versus Tm180 mice.

Figure 3.2. Prominent heart enlargement and fibrosis uncovered for both Tm180 and Tm180/AE3 heart sections. (A) Representative longitudinal heart sections for WT, Tm180 and Tm180/AE3. (B) Representative cross sections for WT, Tm180 and Tm180/AE3. Hearts were obtained from 3-mo old female mice and the 5-µm sections were stained with Masson’s Trichrome for detection of fibrosis.
Immunoblot analysis of NHE1 and AE3 levels in wild-type and mutant hearts

NHE1, which is known to be upregulated in hypertrophic hearts, was increased to the same degree in both single and double mutants (Fig. 3.3A). mRNA encoding the full-length form of AE3 was reported to be upregulated in hypertrophic hearts of the spontaneously hypertensive rat (SHR), with a corresponding reduction in levels of the cardiac AE3 mRNA [98]. Using an antibody that identifies both the full-length and cardiac variants of AE3, high expression of AE3\textsubscript{fl} was observed in WT brain, with no expression of AE3\textsubscript{c} (Fig. 3.3B). In contrast, high levels of AE3\textsubscript{c} but only low levels of AE3\textsubscript{fl} were expressed in ventricles of WT and TM180 single mutant hearts. AE3 expression in ventricles of hypertrophied TM180 hearts was almost identical to that seen in WT ventricles (Fig. 3.3B). Similar levels were seen in whole heart homogenates of WT mice, whereas no expression was detected in AE3-null hearts.
Figure 3.3. NHE1 and AE3 protein expression in ventricles of mutant and wild-type mice. (A) Immunoblot analysis revealed increased expression of NHE1 in ventricles of 3-month-old TM180 transgenic (TG) and TM180/AE3 double mutant (TG/KO) mice; n = 6 male mice of each genotype; *p < 0.001 vs WT. (B) Immunoblotting using an AE3 antibody that identifies both full length (AE3fl) and cardiac (AE3c) forms of AE3 revealed no significant change in ventricles of TM180 single mutant (TG) vs. WT male mice. Note high expression of AE3fl and AE3c in WT brain and whole heart, respectively, and absence of these variants in KO brain and whole heart.
**Force-frequency relationships in vivo**

The data in Fig. 3.1 suggested that loss of AE3 in TM180 mutant mice caused a worsening of the heart failure phenotype. As heart failure is associated with a negative force-frequency response (FFR), *in vivo* pacing studies were performed using WT and both single and double mutant mice. In WT mice, an increase in heart rate from 400 to 550 beats per min (bpm) led to the expected increase in +dP/dt (from 10189 ± 470 to 11986 ± 542; p<0.05), with a slight reduction occurring as heart rate was further increased to 600 bpm (Fig. 3.4A). In contrast, hearts of TM180/AE3 and TM180 mice did not exhibit an increase in contractility as heart rate was increased and could not be efficiently paced beyond 500-550 bpm. TM180/AE3 mice appeared to be less able than TM180 single mutants to achieve or sustain heart rates at the higher frequencies. When the differences between +dP/dt at 400 and 500 bpm were calculated, a positive FFR was apparent in WT mice, whereas both TM180 and TM180/AE3 mutants exhibited a negative FFR (Fig. 3.4B). The results were essentially the same when differences in +dP/dt at 40 mm Hg (dP/dt<sub>40</sub>) between 400 and 500 bpm were calculated (data not shown).
Figure 3.4. TM180 (TG) and TM180/AE3 (TG/KO) mutants exhibit a negative force-frequency response. Hearts of anesthetized surgically-instrumented 3-month-old mice were subjected to atrial pacing beginning at 400 beats per minute (bpm) and contractile parameters were measured. n = 5 WT, 4 TG, and 5 TG/KO mice, with 2 males and either 2 or 3 females of each genotype. WT mice could be paced to 550 and 600 bpm but some TG and TG/KO mice could not. If fewer than 3 mice could achieve a given frequency, such as TG/KO at 550 bpm, the data were not plotted. (A) A positive FFR with respect to maximum $+\text{dP}/\text{dt}$ was observed in WT mice but not in TG and TG/KO mice. (B) Difference in $+\text{dP}/\text{dt}$ at 400 bpm and 500 bpm revealed a negative FFR in TG and TG/KO mice. *p < 0.02 vs WT.
Effects of AE3 ablation on cardiac performance in TM180 mice

Cardiovascular performance of anesthetized WT, TM180, and TM180/AE3 mice was analyzed, with measurements conducted under both basal conditions and following β-adrenergic stimulation via infusion of dobutamine (Fig. 3.5). Previous studies of WT and AE3-null mice using the same protocols showed that loss of AE3 by itself caused no significant changes in heart rate, MAP, or cardiac performance [1]. During β-adrenergic stimulation, heart rate (Fig. 3.5A) was reduced in TM180/AE3 mutants compared with WT and TM180 single mutants (TM180/AE3, 417 ± 15 bpm; TM180, 510 ± 9 bpm; WT, 563 ± 8 bpm; p < 0.01), and MAP was also reduced in double mutants under both basal and stimulated conditions (Fig. 3.5B). Systolic left ventricular pressure was lower in double mutants relative to both TM180 and WT mice under basal conditions (TM180/AE3, 79.8 ± 3.2 mm Hg; TM180, 93.4 ± 3.4 mm Hg; WT, 98.8 ± 4.6 mm Hg; p < 0.05) and after β-adrenergic stimulation (Fig. 3.5C). β-adrenergic stimulation of contractility, as indicated by measurements of maximum +dP/dt and +dP/dt₄₀, and relaxation, as measured by minimum -dP/dt, were significantly reduced in TM180/AE3 mutants relative to both WT and TM180 mice (Fig. 3.5D-F). When differences between values obtained under basal conditions and after maximum β-adrenergic stimulation were calculated, significant differences in stimulation of heart rate, contractility, and relaxation were observed between all three genotypes (Fig. 3.5G-I), with TM180/AE3 double mutants being more severely affected than TM180 single mutants.
Figure 3.5. β–adrenergic stimulation of cardiovascular performance is severely reduced in TM180/AE3 double mutants. Pressure measurements were recorded using transducers in the left ventricle and right femoral artery of anesthetized 2.5-month-old WT, TM180 (TG), or TM180/AE3 (TG/KO) mice of each genotype under basal conditions and in response to β–adrenergic stimulation (intravenous infusion of increasing doses of dobutamine). Heart rate (A), mean arterial pressure (B), systolic left ventricular pressure (C), maximum +dP/dt (D), +dP/dt at 40 mm Hg (E), and minimum -dP/dt (F) are shown for WT, TG, and TG/KO mice. Differences between basal and maximum values during β–adrenergic stimulation are shown for heart rate (G), +dP/dt (H), and -dP/dt (I). n = 7 WT (4 female, 3 male), 9 TG (5 female, 4 male), and 6 TG/KO (4 female, 2 male) mice. *p < 0.05 vs WT, †p < 0.05 vs TG.
Expression of proteins involved in Ca\(^{2+}\) handling

Alterations in proteins involved in Ca\(^{2+}\) handling can have a major impact on cardiac performance. The primary mechanisms for influx and extrusion of Ca\(^{2+}\) across the sarcolemma are the L-type Ca\(^{2+}\) channel (LTCC) and the NCX1 Na\(^+\)/Ca\(^{2+}\) exchanger (Fig. 3.6 and 1.8). Immunoblot analysis revealed increased expression of the \(\alpha2\) subunit of the LTCC in cardiac homogenates of both TM180/AE3 and TM180 mice compared with those of WT mice (Fig. 3.7A). Relative to levels in WT hearts, expression of NCX1 was increased modestly but significantly in TM180 hearts, but not in TM180/AE3 hearts (Fig. 3.7B). Expression of the ryanodine receptor, the Ca\(^{2+}\) release channel of the sarcoplasmic reticulum (SR), was reduced in both mutants (Fig. 3.7C).

Sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase isoform 2a (SERCA2a) is the SR Ca\(^{2+}\) pump, and phospholamban (PLN) is the major regulator of SERCA2a activity [99]. When PLN is in the non-phosphorylated form it inhibits SERCA2a activity, but these inhibitory effects are alleviated when PLN is phosphorylated on Ser16 and/or Thr17 [99]. Immunoblot analysis of cardiac homogenates from WT, TM180, or TM180/AE3 mice revealed no significant differences in either SERCA2a (Fig. 3.7C) or PLN (Fig. 3.7D) expression. However, levels of PLN phosphorylated on Ser16 (Fig. 3.7E) were sharply upregulated in TM180 and TM180/AE3 hearts when compared to WT hearts. Slightly higher levels of phosphorylation were observed in TM180/AE3 hearts than in TM180 hearts, but the differences between double and single mutants were not statistically significant.
Figure 3.6. Schematic diagram showing calcium handling proteins targeted for analysis in this study. Red arrows indicate stimulation of the β–adrenergic receptor pathway (G. Dorn and J. Molkentin, 2004).
**Figure 3.7.** Expression of proteins involved in Ca\(^{2+}\) handling. Immunoblot analysis was performed using homogenates of ventricles from 2.5- or 3-month-old male WT, TM180 (TG), and TM180/AE3 (TG/KO) mice. Representative immunoblots and relative expression levels are shown for the L-type Ca channel, (A), NCX1 Na\(^+\)/Ca\(^{2+}\) exchanger (B), ryanodine receptor (C), SERCA2a Ca\(^{2+}\) pump (D), phospholamban (E), and PLN phosphorylated on Ser16 (PS16) (F). n = at least 6 mice of each genotype, except for panel E, in which n = 3 of each genotype; *p < 0.05 vs WT.
Expression or localization of CamKII and protein phosphatases 1 and 2A

Immunoblot analysis showed that CamKII, which is known to be upregulated in heart failure [100], was increased in cardiac homogenates of both mutants, with slightly higher levels in double mutants (Fig. 3.8A). The catalytic subunit of protein phosphatase 1 (PP1-C), which plays a major role in dephosphorylation of Ser16 of PLN [23], was increased in both mutants when compared to WT controls (Fig. 3.8B), with significantly higher levels in TM180 hearts than in TM180/AE3 hearts. In contrast, no differences in total levels (carboxymethylated and nonmethylated) of the catalytic subunit of protein phosphatase 2A (PP2A-C) in cardiac homogenates were observed between any of the genotypes (Fig. 3.8C). However, the levels of nonmethylated-PP2A-C were significantly increased in cardiac homogenates of both TM180 and TM180/AE3 hearts, with slightly higher levels in TM180/AE3 hearts (Fig. 3.8D), and both total PP2A-C and nonmethylated PP2A-C were increased in the myofibrillar fractions of TM180 and TM180/AE3 hearts (Figs. 3.8E and F).
Figure 3.8. Expression of Ca$^{2+}$-calmodulin-dependent protein kinase II (CaMKII) and catalytic subunits of protein phosphatases PP1 and PP2A (PP1-C and PP2A-C). Immunoblot analysis was performed using homogenates of ventricles (A-D) or myofibrillar fractions (E,F) from 2.5-month-old male WT, TM180 (TG), and TM180/AE3 (TG/KO) mice. Representative immunoblots and relative expression levels are shown for (A) CamKII, (B) PP1-C, (C) total PP2A-C (non-methylated and methylated), (D) non-methylated form of PP2A-C, (E) total (non-methylated and methylated) PP2A-C associated with the myofibrillar fraction, (F) non-methylated PP2A-C associated with the myofibrillar fraction. n = at least 6 male mice of each genotype. *p < 0.05 vs WT; †p < 0.05 vs TG.
The studies in Fig. 3.7 showed that phosphorylation of PLN on Ser16, which is the major mechanism by which β-agonists stimulate contractility [101], is increased 2.2 ± 0.2-fold in TM180 hearts and 2.7 ± 0.4-fold in TM180/AE3 hearts under basal conditions. This suggests that they have a reduced reserve capacity for β-adrenergic stimulation, which could be involved in their impaired cardiac performance in response to dobutamine (Fig. 3.5). To test this hypothesis, we analyzed the phosphorylation of PLN in hearts of WT mice under both basal and stimulated conditions and in mice of all three genotypes after maximum β-adrenergic stimulation.

In a control experiment, we first compared Ser16 phosphorylation under basal conditions in ventricular homogenates from WT mice anesthetized as in Fig. 3.7 with samples prepared using the same surgical and anesthesia conditions used in Fig. 3.5. Ser16 phosphorylation was the same in hearts of avertin-treated or ketamine and inactin-treated surgically-instrumented mice, indicating that the surgical treatment did not affect Ser16 phosphorylation (data not shown). In surgically-instrumented WT mice, dobutamine treatment led to a 2.8-fold increase in phosphorylation of Ser16 (Fig. 3.9A), but only an 18 % increase in phosphorylation of Thr17 (Fig. 3.9B), indicating that Thr17 phosphorylation was near maximum in WT mice under basal conditions. We then compared PLN phosphorylation in WT, TM180, and TM180/AE3 mice after β-adrenergic stimulation. Phosphorylation of Ser16 was only slightly higher in single (p < 0.05) and double (p = 0.12) mutants relative to WT controls (Fig. 3.9C). Given the high levels of Ser16 phosphorylation in mutant hearts under basal conditions (Fig. 3.7E), it is clear that there is a sharp reduction in reserve capacity for Ser16 phosphorylation under maximally stimulated conditions.
conditions. Phosphorylation of Thr17 was essentially the same in WT and TM180 single mutants, but was significantly lower in TM180/AE3 double mutants (Fig. 3.9D).

Figure 3.9. Effects of β–adrenergic stimulation in vivo on phosphorylation of phospholamban in WT and mutant mice. Mice were anesthetized and surgically-instrumented as in Fig. 3. Ventricles were collected under basal conditions or after maximum stimulation with dobutamine, and immunoblot analysis of homogenates was performed using antibodies that recognize phosphoserine 16 (PS16) or phosphothreonine 17 (PS17). (A) PS16 and (B) PS17 levels in WT ventricles under both basal (-dobutamine) and stimulated (+ dobutamine) conditions. (C) PS16 and (D) PS17 levels in WT, TM180 (TG), and TM180/AE3 (TG/KO) ventricles following maximum β–adrenergic stimulation. n = 4 (A and B) or 3 (C and D) 2.5-month-old male mice of each genotype. *p < 0.05 vs WT; †p < 0.05 vs TG.
Effects of AE3 ablation on Ca\(^{2+}\) handling in isolated myocytes of TM180 mice

The above studies showed that TM180 and TM180/AE3 hearts have a reduced reserve capacity for β–adrenergic stimulation of PLN phosphorylation. To determine whether there are differences in Ca\(^{2+}\) handling among the three genotypes, ventricular myocytes were isolated from WT, TM180, and TM180/AE3 mice and Ca\(^{2+}\) transients were analyzed after loading with Fura-2AM. The amplitude of the Ca\(^{2+}\) transient (Table 3.1 and Fig. 3.10A and B) was significantly reduced in myocytes of TM180/AE3 mutants when compared with either TM180 or WT cells, and was modestly reduced in TM180 myocytes relative to WT (340/380nm fluorescence ratios: WT, 0.86 ± 0.04; TM180, 0.78 ± 0.02; TM180/AE3, 0.67 ± 0.04). Moreover, time to 50% decay of the Ca\(^{2+}\) transient (Table 3.1 and Fig. 3.10C) was significantly greater in TM180/AE3 and TM180 myocytes than in WT myocytes (0.315 ± 0.015, 0.291 ± 0.020, and 0.230 ± 0.013 seconds, respectively). Thus, both mutants exhibit impaired Ca\(^{2+}\) handling, with double mutants being more severely affected.

Table 3.1. Calcium transient amplitude in WT, Tm180 and Tm180/AE3 cardiomyocytes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Amplitude (340/380 nm)</th>
<th>t-50% (Sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>75/6</td>
<td>0.86 ± 0.04</td>
<td>0.230 ± 0.012</td>
</tr>
<tr>
<td>Tm180</td>
<td>81/6</td>
<td>0.78 ± 0.02</td>
<td>0.290 ± 0.019</td>
</tr>
<tr>
<td>Tm180/AE3</td>
<td>72/6</td>
<td>0.67 ± 0.04(^\ast),(\dagger)</td>
<td>0.315 ± 0.015</td>
</tr>
</tbody>
</table>

\(^\ast\) p < 0.01 vs. WT; \(\dagger\) p < 0.05 vs. Tm180.
**Figure 3.10.** Analysis of Ca\(^{2+}\) transients in cardiac myocytes from WT and mutant mice. Myocytes were isolated from 3-month-old WT, TM180 (TG), and TM180/AE3 (TG/KO) mice and loaded with Fura-2AM. Ca\(^{2+}\) transients were analyzed during stimulation at 0.5 Hz. (A) Representative tracings of Ca\(^{2+}\) transients for the three genotypes, determined as fluorescence ratios at 340/380 nm. (B) Amplitudes of Ca\(^{2+}\) transients (systolic - diastolic values) for all three genotypes. (C) Time to 50% recovery of the Ca\(^{2+}\) transient (TRC 50%) for all three genotypes. For all three genotypes, n = 6 mice (4 females and 2 males) with 9-16 cells for each mouse. *p < 0.03 vs WT; †p < 0.05 vs TG.
Caffeine-induced Calcium Release

Application of caffeine (10 mM) onto Fura2AM-loaded cardiomyocytes was used to analyze the sarcoplasmic reticulum (SR) calcium loading and release. Caffeine application would induce a prompt release (and complete emptying) of SR calcium content. Our data indicate significantly lower SR Ca\textsuperscript{2+} loading in Tm180 and Tm180/AE3 vs. WT cells [Tm180, 0.82 ± 0.04; Tm180/AE3, 0.81 ± 0.09 and WT, 1.04 ± 0.05; p < 0.05 for both mutants vs. WT cells] (Table 3.2 and Fig. 3.11). These data are in agreement with the calcium transient amplitude results presented earlier which show significantly lower twitches amplitudes in Tm180/AE3, and to a lesser extent in Tm180, cardiomyocytes versus WT cells (Table 3.1 and Fig. 3.10). Hence, the reduced level of SR Ca\textsuperscript{2+} loading would explain, at least partially, the lower Ca\textsuperscript{2+} transient amplitude in both Tm180 and Tm180/AE3 cardiomyocytes. In addition, time to 50% calcium decay from peak (t-50%), which is indicative of NCX1 activity in this case, is significantly longer in both Tm180 and Tm180/AE3 versus WT cells (Tm180, 2.2 ±0.21 sec; Tm180/AE3, 2.18 ± 0.13 and WT, 1.52 ± 0.15; p < 0.05 for both mutants vs. WT cells). These decay data indicate less efficient sarcolemmal Ca\textsuperscript{2+} extrusion via NCX1 in both Tm180 and Tm180/AE3 mutant cardiomyocytes. Paradoxically, the protein level of NCX1 is increased in Tm180, and to a lesser extent in Tm180/AE3, vs. WT cells which would be expected to result in faster Ca\textsuperscript{2+} decay. One explanation for this unexpectedly longer decay in Tm180 and Tm180/AE3 cells is that the cytosolic Ca\textsuperscript{2+} load is exceeding the extrusion capacity of NCX1 in Tm180 and Tm180/AE3. Alternatively, post-translational modification of NCX1 might reduce its activity to a greater extent than the increase in protein level observed in mutant cells.
Table 2.2. Caffeine-induced calcium release in isolated cardiomyocytes from Tm180, Tm180/AE3 and WT mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n (Cells/Hearts)</th>
<th>Amplitude (340/380 nm)</th>
<th>t-50% (Sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>23/7</td>
<td>1.04 ± 0.05</td>
<td>1.52 ± 0.15</td>
</tr>
<tr>
<td>TM180</td>
<td>77/7</td>
<td>0.82* ± 0.04</td>
<td>2.2* ± 0.21</td>
</tr>
<tr>
<td>Tm180/AE3</td>
<td>47/5</td>
<td>0.81* ± 0.09</td>
<td>2.18* ± 0.13</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. WT cells.

Figure 3.11. SR Ca$^{2+}$ load is significantly lower in both Tm180 and Tm180/AE3 cardiomyocytes vs. WT cells. (A) Level of SR Ca$^{2+}$ load in the three genotypes. (B) t-50% decay time. (C) Representative traces for WT, Tm180 and Tm180/AE3 SR Ca$^{2+}$ load in isolated cardiomyocytes. The first few twitch peaks are used to load SR to the steady state, followed by twitch halt for 20 sec and addition of caffeine (arrows) to trigger SR Ca$^{2+}$ release. * p < 0.05 vs. WT levels.
Discussion

Inhibition of Cl⁻/HCO₃⁻ exchange has been proposed as being potentially cardioprotective in hypertrophy, I/R injury, and arrhythmias [4, 17, 20, 95]; however, given the expression of at least four different Cl⁻/HCO₃⁻ exchangers in heart and the absence of isoform-specific inhibitors, the role of individual isoforms is unclear. To begin addressing this issue, we assessed the effects of AE3 ablation on cardiac function and hypertrophy in a mouse model of familial hypertrophic cardiomyopathy [81]. As discussed below, the results indicate that the absence of AE3, rather than providing cardioprotection, leads to more rapid decompensation.

Previous studies indicated that AE3-null mice were healthy [1, 22] and had normal cardiovascular function [1], although they had a modest reduction in heart weight/body weight ratios [1], consistent with the possibility that inhibition of AE3 might reduce hypertrophy. The rationale for a cardioprotective effect is that AE3-mediated Cl⁻/HCO₃⁻ exchange has the potential to operate in concert with the NHE1 Na⁺/H⁺ exchanger [3, 20]. Increased NHE1 activity is known to cause hypertrophy [102] and inhibition of NHE1 is anti-hypertrophic [97], so loss of Cl⁻/HCO₃⁻ exchange that is coupled to NHE1 might be expected to reduce hypertrophy. However, the degree of hypertrophy was the same in TM180 and TM180/AE3 double mutants, and TM180/AE3 mutants progressed much more rapidly to heart failure and death.

As in other models of cardiac hypertrophy [103], NHE1 levels were increased in both TM180 and TM180/AE3 mutant hearts, but the loss of AE3 had no effect on the levels of NHE1. Increased Cl⁻/HCO₃⁻ exchange has been observed in hypertrophied hearts of spontaneously hypertensive rats [21, 98], which also exhibited increased NHE1 activity [21], and it was suggested that increased expression of AE3ᵋ, the full-length variant of AE3, might provide a
balancing Cl⁻/HCO₃⁻ exchange activity that might facilitate increased NHE1 activity [20]. However, mouse ventricle contains almost no AE3ₐ mRNA [91]. Furthermore, immunoblot analysis showed high levels of AE3_c in both WT and hypertrophied TM180 hearts but only trace levels of AE3ₐ protein, and no change in expression of either AE3 variant was observed in TM180 hearts. These data suggest that AE3ₐ does not play a major role in cardiac muscle of either WT or TM180 mice and that the enhanced rate of decompensation in TM180/AE3 double relative to TM180 single mutants is due to the loss of AE3_c. Unlike mouse heart, however, ventricle tissue from failing human heart expressed similar levels of mRNA for both AE3ₐ and AE3_c [4]. Thus, there are major species differences in expression of the two AE3 variants and it is possible that coupling between NHE1 and AE3ₐ occurs in human heart.

Although studies using an inhibitory antibody suggest that AE3 balances the alkalinizing activity of NHE1 [9], the current results provide no evidence that expression of NHE1 and AE3 are coordinately regulated in mouse heart. When isolated myocytes are analyzed in Hepes buffer or in the presence of inhibitors of Cl⁻/HCO₃⁻ exchange, NHE1-mediated Na⁺/H⁺ exchange leads to an increase in pHᵢ [21, 102]. However, this change in pHᵢ does not occur if cells are maintained in the absence of inhibitors or in buffer containing CO₂/HCO₃⁻, thus indicating that NHE1 activity is balanced by Cl⁻/HCO₃⁻ exchange [21]. Although AE3 remains a candidate to mediate this activity, one or more of the other Cl⁻/HCO₃⁻ exchangers could also be involved. AE2 is a reasonable candidate, as functional coupling of NHE1 and AE2 on basolateral membranes of mouse colonic epithelium has been shown to support cAMP-stimulated anion secretion [40], and both NHE1 and AE2 are sensitive to pHᵢ [104, 105] and can function in concert to regulate cell volume [106, 107]. NHE1 and AE1 are both present in intercalated discs [108, 109], consistent with possible coupling between these transporters in this location. Finally,
the very high levels of Slc26a6 in heart [92] make it a good candidate to balance at least some of
the activity of NHE1.

*In vivo* force-frequency relationships were positive in WT mice, but negative in both
mutants, consistent with the negative FFR typically observed in human heart failure [110]. There
was little indication of a difference in FFR between single and double mutants as pacing
frequency was increased to 500 bpm; however, TM180/AE3 double mutants had more
arrhythmic events at the higher pacing frequencies and exhibited greater failure in achieving the
desired heart rate as pacing frequencies were increased beyond 500 bpm. In addition, maximal
heart rates following β-adrenergic stimulation were substantially lower in TM180/AE3 mutants
than in TM180 and WT mice. These data suggest that the loss of AE3 leads to a general
reduction in the ability of TM180 mutants to achieve or maintain elevated heart rates, although it
should be noted that loss of AE3 alone does not cause a significant reduction in heart rate under
either basal or stimulated conditions [1].

Basal contractility was normal in 2.5-month-old single and double mutants and basal
relaxation, as indicated by -dP/dt, was similarly reduced in both mutants. However, TM180/AE3
double mutants exhibited a severe reduction in β-adrenergic responses. The data for WT and
TM180 mice are consistent with a previous study using the isolated work-performing heart [81].
In that study, hearts from 2.5-month-old TM180 mice exhibited normal +dP/dt under basal
conditions but had sharply reduced absolute values of -dP/dt compared to WT hearts, consistent
with a relaxation defect caused by the increased Ca^{2+}-sensitivity of the TM180 contractile
apparatus [81], and β-adrenergic responses were reduced to about the same degree as in the
current study. When isolated hearts from 4.5-month-old TM180 mice were analyzed,
β-adrenergic stimulation of both contraction and relaxation were lost [81], as seen in the current study with 2.5-month-old TM180/AE3 mutants. For both single and double mutants, loss of β-adrenergic stimulation occurs at about the age that a significant incidence of death from heart failure begins to occur. Previous studies showed that loss of AE3 alone or in combination with loss of NKCC1 does not affect responses to dobutamine [1]. These and the current observations suggest that the loss of β-adrenergic responses in young TM180/AE3 mice is due primarily to the TM180 mutation rather than to the loss of AE3 per se, but with more rapid decompensation and heart failure occurring in TM180 mice that also lack AE3.

Phosphatase activity is elevated in end-stage heart failure in humans [111], and cardiac-specific over-expression of PP1 leads to impaired contractility and dilated cardiomyopathy [112]. In AE3-null mice, which appeared outwardly normal, expression of PP1 was elevated and methylation of PP2A was altered [1], supporting the possibility that alterations in these phosphatases, both of which can affect β-adrenergic responses [112, 113], might contribute to the more rapid development of heart failure in TM180/AE3 double mutants. However, both single and double mutants exhibited increases in PP1, non-methylated PP2A, and both total and non-methylated PP2A in the myofibrillar fraction, indicating that differences in phosphatase levels are not responsible for the worsening heart failure in TM180/AE3 mice. Expression of CamKII, which is often upregulated and activated in heart failure [114] and mediates some of the effects of β-adrenergic signaling [115], was also increased. CamKII levels were slightly higher in TM180/AE3 hearts, suggesting that it contributes to the more rapid decompensation.

Phosphorylation of PLN on Ser16, a major mechanism by which β-adrenergic signaling stimulates cardiac performance, was sharply increased in both mutants under basal conditions.
The increased phosphorylation of PLN likely contributes to maintenance of cardiac performance in mutant mice under basal conditions and is consistent with increased catecholamine stimulation that occurs in heart failure. Failing hearts of muscle-LIM-protein (MLP)-null mice exhibited a similar increase in PLN phosphorylation [116], which the investigators noted would lead to a reduced reserve capacity for enhanced Ca^{2+} handling in response to increased frequency or adrenergic stimulation. Analysis of hearts from surgically instrumented mice revealed a 2.8-fold increase in phosphorylation of Ser16 in WT hearts in response to \( \beta \)-adrenergic stimulation. Under maximally stimulated conditions, however, similar levels of Ser16 phosphorylation were observed in all three genotypes. Given the large differences in Ser16 phosphorylation between WT and mutant hearts under basal conditions (2.2-fold and 2.7-fold higher in TM180 and in TM180/AE3 hearts, respectively), it is clear that the mutants have a reduced reserve capacity for PLN phosphorylation on Ser16, as suggested for MLP-null mice [116].

Phosphorylation of PLN on Thr17 in WT hearts was near maximum levels under basal conditions (with heart rates at ~350 bpm), and little additional phosphorylation occurred in response to dobutamine (with heart rates at ~560 bpm). Thr17 phosphorylation in TM180 hearts after \( \beta \)-adrenergic stimulation was the same as in WT hearts and levels in TM180/AE3 hearts were slightly reduced. Previous studies have shown that Thr17 phosphorylation responds to increased frequency, but not to \( \beta \)-adrenergic stimulation, whereas the opposite is true for phosphorylation of Ser16 [117]. Thus, the modest reduction in phosphorylation of Thr17 in TM180/AE3 hearts relative to WT or TM180 hearts, which might be due in part to the reduced heart rate, is unlikely to contribute to the impaired \( \beta \)-adrenergic response.
Ca$^{2+}$-handling was impaired in isolated myocytes from both mutants, with reduced Ca$^{2+}$ transients and prolonged decay times; however, it was more severely affected in TM180/AE3 myocytes. These alterations may be due in part to increased myofibrillar Ca$^{2+}$ sensitivity resulting from the TM180 mutation, as the sensitized myofilaments can reduce the amplitude by providing increased Ca$^{2+}$ buffering power and reduce the decay rate by slowing the dissociation of Ca$^{2+}$ [118]. Expression of the L-type Ca$^{2+}$ channel was higher in both mutants, which might be expected to increase, rather than decrease, the amplitude of the Ca$^{2+}$ transient. However, one of the most striking effects on Ca$^{2+}$ handling proteins was a reduction in levels of the ryanodine receptor, consistent with the reduced Ca$^{2+}$ transient amplitudes. The expression of SERCA2a in heart is generally decreased in both human heart failure and in animal models of heart failure [119] and is often considered a major contributing factor to reductions in Ca$^{2+}$ transients [119, 120]. However, SERCA2a levels were not significantly reduced in TM180 and TM180/AE3 hearts at 2.5-3 months of age. Although the greater deficit in Ca$^{2+}$ handling in TM180/AE3 mutants is undoubtedly involved in their more rapid progression to heart failure, impaired Ca$^{2+}$ handling occurs commonly in heart failure [121] and many of the changes were similar in single and double mutants. Thus, it is likely that the greater impairment of Ca$^{2+}$ handling in myocytes from TM180/AE3 mice is secondary to their more rapid progression to heart failure rather than being a direct consequence of the loss of AE3.

In conclusion, loss of AE3 caused no lessening of hypertrophy in the TM180 heart but led to more rapid decompensation and heart failure. This suggests that AE3 would not be an appropriate target for inhibitory drug therapy, at least in cardiomyopathies similar to those involving the Glu180 to Gly mutation in β–tropomyosin [79]. Although the data do not rule out the possibility that inhibition of AE3 might be useful in other conditions, the absence of
protective effects of AE3 ablation on I/R injury in a Langendorff heart model [1] and the negative effects of AE3 ablation on cardiac performance in mice lacking the NKCC1 Na⁺-K⁺-2Cl⁻ cotransporter [1] also argue against a therapeutic value of AE3 inhibition. Finally, the reduced ability of TM180/AE3 mutants to achieve or maintain higher heart rates during pacing suggests that it also might not be an appropriate target for anti-arrhythmic therapy. Rather, the data indicate that AE3-mediated Cl⁻/HCO₃⁻ exchange activity, which appears to be dispensable in a healthy heart [1], contributes to better preservation of cardiac function during heart failure.
Chapter 4: Tm54/AE3 Mouse Model

Summary

AE3 is an integral plasma membrane protein with high expression level in cardiac tissue. To further analyze the role of AE3 in the heart we crossed AE3 knockout (KO) mice with a transgenic (Tm54) mouse model of dilated cardiomyopathy carrying a Glu54Lys mutant of α–tropomyosin. AE3 ablation reduced cardiac hypertrophy significantly in this model, with a heart weight/body weight ratio (mg/g) of 6.0 in Tm54 and 5.6 in Tm54/AE3 mice (p = 0.018). However, both of these mutant genotypes showed similar degrees of heart enlargement vs. their respective wild-type (WT) or AE3-null controls (p < 0.0001). In addition, levels of β–myosin heavy chain (β–MHC) were significantly increased in cardiac homogenates of both Tm54 and Tm54/AE3 double mutant mice vs. WT, although the level was significantly lower in Tm54/AE3 when compared with Tm54. Analysis of heart function revealed severely depressed contractility and relaxation in both mutant genotypes when compared with WT mice, and there was no improvement in cardiac function in Tm54/AE3 vs. Tm54 mice. In contrast, analysis of calcium transients in isolated cardiomyocytes revealed significantly higher amplitudes in Tm54/AE3 cells vs. cells from Tm54 or WT animals. Analyses of calcium handling proteins in the heart showed no significant differences between genotypes in levels of the SR calcium pump (SERCA2a) or phosphorylated phospholamban. The levels of L-type calcium channel were significantly increased in both Tm54 and Tm54/AE3 vs. WT cardiac homogenates, but no differences were uncovered between Tm54 and Tm54/AE3. Finally, mean values for levels of the Na⁺/Ca²⁺ exchanger (NCX1) were increased in Tm54 and Tm54/AE3 vs. WT, but were significant only in
Tm54 cardiac homogenate. Overall, these data show that the loss of AE3 has a major impact on calcium handling in cardiomyocytes of the Glu54Lys α–tropomyosin cardiomyopathy model.

Introduction

AE3 is the major anion exchanger in the heart, which also expresses three other chloride-bicarbonate exchangers, AE1, AE2 and PAT1 [11, 12, 14, 92, 122, 123]. Each transporter mediates the efflux of bicarbonate from the cell and influx of chloride. Given the fact that bicarbonate is a base equivalent, it is anticipated that AE3 activity would give rise to acid loading effects. However, AE3 is hypothesized to work in concert with the sodium-proton exchanger (NHE1), which displays an acid extruding activity, to maintain a pH$_{1}$-neutral sodium loading activity [3]. As such, the working hypothesis is that AE3 operating in concert with NHE1 will lead ultimately to sodium loading of cardiac myocytes. Sodium loading on its own is expected to have detrimental effects on cardiac myocytes through its effect on the driving force of the sodium-calcium exchanger (NCX1), and the subsequent calcium loading with the potential for contracture injury [29]. In addition, this anticipated calcium loading is expected to have further effects on the rhythm of cardiac cells leading to arrhythmia and perturbations of heart rate regulation [85]. Chloride influx via AE3 is also suggested to affect intracellular ion homeostasis and specifically its contribution to the triggering of action potentials [4].

Keeping in mind all these proposed effects of chloride/bicarbonate exchange in cardiac myocytes, it is possible to imagine a beneficial outcome of AE3 ablation. Indeed, it has been suggested previously that AE3 ablation would be a desirable target for cardioprotective effects [4]. However, our earlier findings of AE3 ablation in the Tm180 mouse model of hypertrophic
cardiomyopathy are discouraging with regard to cardioprotective effects (Chapter 3). Indeed, the mice display a more severe disease phenotype and die earlier due to heart failure.

In this chapter, we are using an entirely different mouse model exhibiting dilated cardiomyopathy (DCM), which has a very different phenotype than the Tm180 model, which developed a hypertrophic cardiomyopathy (HCM). Hence, these are two entirely distinct heart disease entities [81, 82]. Moreover, these two models exhibit sharp differences with respect to calcium sensitivity of the myofilaments. Tm180 has a high calcium sensitivity, whereas Tm54 exhibits low calcium sensitivity and, as discussed above, AE3 activity is inherently correlated with calcium homeostasis through its putative effect on sodium loading. To further investigate the cardioprotective effects and functional ramifications of AE3 ablation this study was initiated using the Tm54 model of dilated cardiomyopathy.

The overall findings reveal very different effects of AE3 ablation compared with our earlier observations in the Tm180 model. In the current study using the Tm54 mouse model, AE3 ablation did not have the detrimental effects seen in Tm180/AE3 double mutants. Indeed, the Tm54 single mutant and Tm54/AE3 double mutant revealed very similar disease phenotypes, with both displaying severely depressed heart function in comparison with WT animals. As in the Tm180 model of hypertrophic cardiomyopathy, AE3 ablation did not exhibit cardioprotective effects in the Tm54 model of DCM. Unexpectedly, however, calcium imaging studies using isolated cardiomyocytes showed significantly higher levels of calcium transient amplitude in Tm54/AE3 versus Tm54 cardiomyocytes. As such, AE3 ablation has a very distinct effect on calcium cycling in the Tm54 model, albeit not alleviating the disease phenotype. The
improvement in calcium cycling, with no improvement in disease progression, suggests that the increased calcium transients may counteract possible negative effects of AE3 ablation.

Results

Histology

Longitudinal and cross sections of the hearts were prepared from 12-month old animals, and sections were stained with both H&E and Masson’s trichrome. Both Tm54 and Tm54/AE3 hearts displayed prominent dilatation (an increase in the inner diameter of left ventricles) when compared with WT animals (Fig. 4.1). This finding is in accordance with the original description of the Tm54 mouse model, which displays cardiac dilatation and other hallmarks featuring dilated cardiomyopathy [82]. The occurrence of the same changes in heart sections from Tm54/AE3 double mutant mice suggests that AE3 ablation has no gross effect on the degree of dilatation and cardiac remodeling in Tm54 mice. In addition, the overall enlargement of the myocardium is obvious in both Tm54 and Tm54/AE3 histology sections, which is in agreement with the H/BW analysis presented in the previous section. Finally, the level of fibrosis (detected by Masson’s trichrome staining) is markedly higher in both Tm54 and Tm54/AE3 sections vs WT sections. Careful inspection suggests that Tm54 hearts might have higher level of fibrosis when compared with heart sections prepared from Tm54/AE3 mice; however, no quantification was obtained to confirm this observation.
Figure 4.1. Tm54 and Tm54/AE3 heart sections display prominent dilatation with a marked increase in fibrosis in comparison with WT sections. (A) Longitudinal and (B) Cross sections of heart were stained with Masson’s trichrome. Sections were prepared from 12-month old male animals.
Cardiac Hypertrophy and levels of β–MHC

The degree of cardiac hypertrophy was obtained by calculating the heart weight to body weight ratio. Our results indicate that both Tm54 and Tm54/AE3 double mutant mice developed a very significant enlargement of the heart relative to the body weight (H/BW) when compared with WT animals (Fig. 4.2).

At the age of 2.5 months old, Tm54 mice showed a H/BW ratio of 6.0 ± 0.09 (mg/g of bodyweight), whereas Tm54/AE3 mice had a ratio of 5.6 ± 0.1 and WT mice had a ratio of 4.9 ± 0.08. The difference between Tm54, and Tm54/AE3, versus WT animals is very significant (p < 0.0001). Interestingly, the degree of cardiac hypertrophy is also significantly different between Tm54 and Tm54/AE3 mice, with the later showing reduced cardiac enlargement (p < 0.05). The same effect was also observed between WT and AE3 KO mice, with a significantly lower H/BW ratio in AE3 KO vs. WT animals (Fig. 4.2A). However, the difference in H/BW ratio between Tm54 and Tm54/AE3 mice was nonsignificant when the measurements were made using older animals (5 months old) (Fig. 4.2B). Again, the increase in H/BW ratio is consistently very significant in older mutant mice when compared with WT animals (p < 0.001).

Overall, these findings indicate that AE3 ablation per se might have a modest lowering effect on the H/BW ratio specifically in younger animals (2.5 months). However, this effect is not coherently correlated with the pathological heart enlargement since the reduction that could be attributed to AE3 ablation was observed on both the cardiomyopathy background (Tm54/AE3 vs. Tm54) and on the AE3-null background (AE3 KO vs WT).
In addition, the level of β–MHC, one of the fetal genes that are reactivated under cardiac stress conditions, was analyzed by Western blotting. Our data show that β–MHC levels are sharply increased in both Tm54 and Tm54/AE3 vs. WT heart homogenates, reflecting the stress status experienced by these hearts (Fig. 4.2C). Interestingly, the level of β–MHC is also significantly different between Tm54 and Tm54/AE3 hearts, with higher levels in Tm54 when compared with Tm54/AE3 hearts (Fig. 4.2D). This might suggest a reduced level of cardiac stress in Tm54/AE3 hearts when compared with those of Tm54 animals.

**Figure 4.2.** Cardiac hypertrophy and β–MHC are significantly increased in Tm54 and Tm54/AE3 vs. WT animals. (A) Heart-to-body weight (H/BW) ratio at 2.5 mo, (B) H/BW ratio at 5 mo, (C) Immunoblot for β–MHC with GAPDH serving as loading control, (D) Densitometry of the β–MHC. (n = 6-20 mice for the H/BW analysis and n = 6 for the immunoblot). * p < 0.05 vs. WT animals; † p < 0.05 vs. Tm54.
Heart Function Analysis

Cardiac function was analyzed by three different approaches to answer overlapping and distinct questions pertinent to heart function and/or structure.

M-mode Echocardiography

Transthoracic echocardiography is a noninvasive approach and was performed to obtain both functional and structural cardiac data for Tm54 and Tm54/AE3 versus WT animals. Structurally, our data indicate that Tm54 and Tm54/AE3 mice, as expected, develop a significant dilatation vs. WT animals as indicated by the higher values for left ventricular end-systolic dimension (LVESD) and left ventricular end-diastolic dimension (LVEDD) (p < 0.01 for both Tm54 and Tm54/AE3 mice vs. WT animals) (Table 4.1). However, the level of dilatation is the same for both Tm54 and Tm54/AE3, consistent with our findings presented in the histology section (see above).

Functional parameters, namely, percent of fractional shortening (FS %) and percent of ejection fraction (EF %), show similarly compromised heart function for both Tm54 and Tm54/AE3 vs WT mice. For instance, the level of fractional shortening for Tm54 and Tm54/AE3 mice are 21.2 ± 2.2 % and 21.4 ± 1.4 %, respectively, versus a value of 30.4 ± 1.8% for WT animals [p < 0.01 for both Tm54 and Tm54/AE3 vs. Wt for both FS % and EF %] (Table 4.1).

In summary, the echocardiography data revealed similar structural and functional alterations for both Tm54 and Tm54/AE3 hearts that are statistically significant when compared with hearts from WT animals.
Table 4.1. Summary data from Doppler echocardiography analysis showing significantly larger diameters of heart chambers during diastole and systole, LVEDD and LVESD, respectively (indicators of dilatation) for both Tg (Tm54) and TgKo (Tm54/AE3) mice vs. Wt animals. The two functional parameters (FS%, percent of fractional shortening and EF%, percent of ejection fraction) were also reduced similarly in Tg and TgKo mice vs. Wt animals. 5-6 male mice at the age of 5 months were used for this analysis.

<table>
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<tr>
<th></th>
<th>Wt</th>
<th>Tg</th>
<th>Tgko</th>
<th>P-value</th>
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<tbody>
<tr>
<td></td>
<td>Mean (± sem)</td>
<td>Mean (± sem)</td>
<td>Mean (± sem)</td>
<td>Tg vs. wt</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.96 ± 0.07</td>
<td>4.74 ± 0.19</td>
<td>4.40 ± 0.10</td>
<td>0.005</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.75 ± 0.08</td>
<td>3.75 ± 0.24</td>
<td>3.45 ± 0.08</td>
<td>0.006</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>571 ± 28</td>
<td>504 ± 6</td>
<td>535 ± 17</td>
<td>0.029</td>
</tr>
<tr>
<td>LVM (mg)</td>
<td>142 ± 9</td>
<td>160 ± 17</td>
<td>153 ± 10</td>
<td>0.408</td>
</tr>
<tr>
<td>FS (%)</td>
<td>30 ± 2</td>
<td>21 ± 2</td>
<td>21 ± 1</td>
<td>0.011</td>
</tr>
<tr>
<td>EF (%)</td>
<td>51 ± 2</td>
<td>38 ± 4</td>
<td>38 ± 2</td>
<td>0.013</td>
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</table>
In vivo Analysis of Cardiac Function

For this experiment, a high-fidelity pressure transducer was introduced directly into the left ventricle for mice undergoing complete anesthesia. Femoral vein catheterization was used to introduce different doses of β-agonist (dobutamine) whereas femoral artery catheterization was used for the measurements of mean arterial pressure (MAP).

Our data show no difference in the heart rate among the three genotypes (Tm54, Tm54/AE3 and WT) either under baseline conditions or after β-adrenergic stimulation. In contrast, the pressure parameters, MAP and LVP, revealed significant differences for both Tm54 and Tm54/AE3 vs. Wt animals both at baseline and at most doses of β-agonist (Fig. 4.3). No difference in MAP was observed between Tm54 and Tm54/AE3 animals under basal conditions, although significant differences in MAP were observed at some doses of β-agonist.

Importantly, analysis of contractility (+dP/dt) and relaxation (-dP/dt) indices revealed very significant differences between the two groups of mutant mice versus WT animals (Fig. 4.3). These differences were observed at both baseline and after different doses of dobutamine (Fig. 4.3). For example, the baseline level in +dP/dt show a contractility rate of 9974 ± 783 mm Hg/s for WT animals, whereas it is 3838 ± 366 mm Hg/s for Tm54 and 3929 ± 859 mm Hg/s for Tm54/AE3 mice [p < 0.01 for both mutants vs. Wt]. In addition, no differences were observed between the two mutant groups even when the +dP/dt was normalized to MAP of 40 mm Hg (dP/dt_{40}). The result for dP/dt_{40} is very similar between Tm54 and Tm54/AE3 with the only consistent significant differences occurring in comparisons with WT animals (Fig. 4.3).
Force Frequency Analysis and Cell Mechanics

The third approach used to analyze cardiac function in this project was analysis of the force frequency response. In these experiments the heart was extrinsically stimulated (by electric

Figure 4.3. In vivo analysis of cardiac performance showing similarly depressed heart function at baseline and after β-AR stimulation for Tm54 and Tm54/AE3 WT animals. (A) Heart rate, (B) Mean arterial pressure (MAP), (C) Left ventricular pressure (LVP), (D) Maximal rate of contractility (\(+dP/dt\)) , (E) Maximal rate of relaxation (\(-dP/dt\)) and (F) Normalized contractility at 40 mm Hg MAP (\(dP/dt_{40}\)). (N=WT. 5 mice: Tm54. 5 mice and Tm54/AE3. 4 mice).

Force Frequency Analysis and Cell Mechanics

The third approach used to analyze cardiac function in this project was analysis of the force frequency response. In these experiments the heart was extrinsically stimulated (by electric
pulses) to increase its frequency (beats per minute, BPM), and subsequently to analyze the capacity of the different hearts to increase contractility; the so-called force-frequency response (FFR). It is well established that failing hearts, especially in large mammals, have defective FFR with either inability to maintain a positive force-frequency response or in worse cases to display a negative FFR [124].

In concordance with data presented above, mutant animals from both groups showed similar FFRs which, indeed, are severely compromised, with flat lines across the different frequency points reflecting a blunted force-frequency response (Fig. 4.4). Also a trend of negative FFR can be recognized at the end of the frequency scale for both Tm54 and Tm54/AE3 mice. In contrast, WT animals displayed a progressively increasing contractility at increasing heart rates (Fig. 4.4). The same observation was recognized for -dP/dt (relaxation) with significant differences between WT animals and the two mutant groups. Once again the Tm54 and Tm54/AE3 animals displayed similar force-frequency responses with no significant differences at all of the frequency points show significant differences vs. WT animals. (n = 4,WT; 4, Tm54; 3, Tm54/AE3).

**Figure 4.4.** In vivo Force-Frequency analysis showing severely depressed response in both contractility (A) and relaxation (B) for Tm54 and Tm54/AE3 vs. WT animals. All of the frequency points show significant differences vs. WT animals. (n = 4,WT; 4, Tm54; 3, Tm54/AE3).
differences across the different frequency points.

Analysis of contractility in isolated ventricular cardiomyocytes revealed unexpectedly similar levels of fractional shortening (FS%) in the three groups (Fig. 4.5 and Table 4.2). Similar observations were also made for the cell mechanic indices (rate of contraction, +dL/dt, and rate of relaxation, –dL/dt). Indeed, both the Tm54 and Tm54/AE3 cells revealed slightly enhanced +dL/dt and -dL/dt, although it was not significant relative to WT cardiomyocytes. Hence, it is conceivable that the severely compromised cardiac function found by echocardiography, intraventricular pressure measurements during β-adrenergic stimulation, and FFR analysis, is due essentially to the dilatation and heart remodeling rather than to changes in cardiomyocyte contractility.

![Figure 4.5](image)

**Figure 4.5.** Cell contractility and kinetics show similar levels in the three genotypes. (A) Representative traces of cell mechanics for the three genotypes. (B) Percent of fractional shortening (FS %). (C) Rate of contraction (+dL/dt). (D) Rate of relaxation (-dL/dt). [WT, n = 3 hearts (33 cells); Tm54, n = 3 hearts (33 cells); Tm54/AE3, n = 3 hearts (31 cells)].
Calcium Studies

Calcium studies, including transient analysis and caffeine-induced calcium release, are accomplished by using freshly prepared cardiomyocytes which were preloaded initially with the calcium sensitive dye Fura2-AM. All of the calcium amplitude data below are presented as fluorescent ratios of excitations at 340 nm divided by 380 nm ($R_{340/380}$), which essentially represent the calcium released from the SR after pacing relative to the baseline noise.

Calcium Transients

Calcium transient data were obtained by electrically stimulating cardiomyocytes at a pacing frequency of 0.5 Hz. The data uncovered a significant increase in the amplitude of the calcium transient for Tm54/AE3 double mutant cells, whereas both WT and Tm54 cardiomyocytes show similar levels. The calcium transient amplitudes for WT and Tm54 are $0.80 \pm 0.05$ and $0.84 \pm 0.08$, respectively, versus a value of $1.07 \pm 0.04$ for Tm54/AE3 cardiomyocytes [$p < 0.05$ for Tm54/AE3 vs. the other two groups]. Fig. 4.6 and Table 4.3 summarize calcium transient data along with representative traces for the three genotypes. Given the almost exact phenotype and disease severity of Tm54 and Tm54/AE3 animals, these data were surprising. Hence, our expectation is that the increase in calcium transient amplitude in

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>FS %</th>
<th>+dL/dt (μm/s)</th>
<th>-dL/dt (μm/s)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>3 (33 cells)</td>
<td>14.9 ± 0.2</td>
<td>128 ± 6</td>
<td>104 ± 8</td>
</tr>
<tr>
<td>Tm54</td>
<td>3 (33 cells)</td>
<td>13.2 ± 1</td>
<td>143 ± 4</td>
<td>123 ± 12</td>
</tr>
<tr>
<td>Tm54/AE3</td>
<td>3 (33 cells)</td>
<td>14.3 ± 0.4</td>
<td>136 ± 7</td>
<td>137 ± 19</td>
</tr>
</tbody>
</table>
Tm54/AE3 might be an adaptive mechanism that prevents a more severe phenotype in the double mutant animals.

In addition, analyses of the calcium transient decay show a significantly faster removal of calcium in Tm54 cardiomyocytes when compared with WT cells (Fig. 4.5). Interestingly, the calcium decay is slightly, but not significantly, faster in Tm54 relative to that uncovered in Tm54/AE3. This might be attributed partially to the higher calcium amplitude in Tm54/AE3 vs. Tm54. Moreover, Western blot analysis (see below) supports this observation of faster calcium decay in Tm54 as the level of NCX1 is also increased in Tm54, but not AE3/Tm54, cardiac homogenate.

**Figure 4.6.** Calcium transient amplitude is significantly increased in Tm54/AE3 cardiomyocytes. (A) Calcium transient amplitude in the three genotypes as indicated in the legends, (B) Time to 50% decay from the peak (t-50%) is significantly shorter in Tm54 and Tm54/AE3 cardiomyocytes, (C) Representative calcium transient traces for the three genotypes. [WT, n = 7 hearts (124 cells); Tm54, n = 5 hearts (82 cells) and Tm54/AE3, n = 6 hearts (125 cells)]. * P < 0.01 vs. Wt; † P < 0.01 vs. Tm54.
Table 4.3. Twitch calcium-transient data

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n (cells)</th>
<th>Amplitude (340/380 nm ratio)</th>
<th>t-50% (Sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7 (124)</td>
<td>0.8 ± 0.05</td>
<td>0.238 ± 0.01</td>
</tr>
<tr>
<td>Tm54</td>
<td>5 (82)</td>
<td>0.84 ± 0.08</td>
<td>0.193 ± 0.01†</td>
</tr>
<tr>
<td>Tm54/AE3</td>
<td>6 (125)</td>
<td>1.07 ± 0.04 *</td>
<td>0.206 ± 0.01</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. WT and Tm54; †p <0.05 vs. WT

Caffeine-induced Calcium Release

A major source for either the increase or decrease in calcium transient amplitude is higher or lower SR Ca\(^{2+}\)-loads, respectively. Caffeine-induced calcium release from the SR is accomplished through activation of SR calcium-release channels (RyR2). Our results revealed the same level of SR calcium-loading in cardiomyocytes prepared from Tm54/AE3 double mutant mice and WT cells. In contrast, the level in Tm54 cardiomyocytes is lower than in WT and Tm54/AE3 cells, but the differences were not significant. The results indicate a value of 1.09 ± 0.1 for Tm54/AE3 cells and 1.04 ± 0.04 for WT cells, whereas Tm54 cells show a value of 0.89 ± 0.05 [p = 0.06 for Tm54 vs. WT and p = 0.7 for Tm54/AE3 vs. Tm54] (Fig. 4.7 and Table 4.4). The data suggest that progression of the Tm54 cardiomyopathy leads to reduction in SR Ca\(^{2+}\) load vs. WT cells. This reduction is reversed by AE3 ablation giving rise to WT levels of SR Ca\(^{2+}\) load in Tm54/AE3 double mutant cells. However, Tm54/AE3 double mutant cells show more efficient release of SR Ca\(^{2+}\) than WT cells during calcium transient analysis using 0.5 Hz pacing rate (see above). In addition, calcium decay analysis indicates a slower decay, albeit not significant, for both Tm54 and AE3/Tm54 cells versus cells from Wt animals [Wt, 1.59 ± 0.2 sec; Tm54, 1.64 ± 0.06 sec) and AE3/Tm54, 1.67 ± 0.1 sec]; the differences were not significant]. In caffeine-induced calcium release experiments like this one, and since the SR
loading is inapplicable due to the chronic activation of RyR2 by caffeine application, the decay is due almost solely to the NCX1 activity in the sarcolemma with no role attributed in this case to the SR calcium pump (SERCA2a).

In summary, Tm54/AE3 cardiomyocytes display significantly higher calcium transient amplitudes compared with the other two groups. Obviously, the increase in Ca$^{2+}$ transient amplitude in Tm54/AE3 is partially attributed to the higher SR Ca$^{2+}$-loading as presented above.

*Figure 4.7. Analysis SR calcium load by caffeine-induced calcium release. (A) SR calcium load is not different statistically across the three genotypes, although a trend toward a lower load in Tm54 cells was observed. (B) Decay of calcium after caffeine application is slightly longer, although not significant, in both Tm54/AE3 vs. WT cells. (C) Representative traces for the three genotypes. Vertical arrows indicate the application of caffeine. [WT, n = 7 hearts (23 cells); Tm54, n = 5 hearts (41 cells) and Tm54/AE3, n = 6 hearts (52 cells)].*
Western Blot Analysis

Levels of AE3 in Tm54 Heart Homogenates

AE3 ablation and its effect in the Tm54 cardiomyopathy mouse model is the focus of this project. As such, we analyzed the levels of AE3 in Tm54 hearts to see how it changed with respect to WT levels. Interestingly, our results show a significant increase in AE3, and specifically the cardiac isoform, in Tm54 vs. WT cardiac homogenates (an increase of $36 \pm 0.1\%$ vs. WT; $p < 0.05$) (Fig. 4.8). It would be interesting to understand whether this increase in AE3 levels is adaptive or maladaptive with respect to the cardiomyopathy development in Tm54 mice.
Levels of NHE1

Due to its suggested role in cardiac hypertrophy and a presumed coupling with AE3, NHE1 levels were evaluated in the three genotypes. NHE1 expression was significantly increased in both the Tm54 and AE3/Tm54 mutants with values of 143 ± 6% and 135 ± 10%, respectively, relative to the amount found in WT heart homogenates (p < 0.05) (Fig. 4.8). The relatively higher levels of NHE1 in Tm54 vs. Tm54/AE3 hearts, although modest, might correlate with the presence of AE3 expression in these hearts, which indeed is overexpressed versus WT as shown above.

Figure 4.8. AE3 is significantly increased in Tm54 vs. WT heart homogenates whereas NHE1 is significantly increased in Tm54 and Tm54/AE3 vs. WT heart homogenates. (A) AE3 immunoblot and the corresponding densitometry analysis, (B) Levels of NHE1 and the corresponding densitometry analysis. GAPDH and s-actin serve as loading controls. (n = 6 heart homogenates for each genotype). * p < 0.05 vs. WT levels.
**Calcium Handling Proteins**

Given the findings in Tm54/AE3 double mutant cells that the Ca\(^{2+}\)-transient amplitude is significantly increased and that the SR calcium load shows a trend to higher levels vs. both WT and Tm54 cells, we analyzed the proteins involved in calcium cycling in cardiac myocytes. These investigations were accomplished in an attempt to find a plausible molecular explanation for the divergent calcium cycling observations mentioned above.

**LTCC Levels and activity**

The voltage-gated L-type calcium channel (LTCC) is the protein responsible for triggering calcium-induced calcium release (CICR), and hence the release of calcium from the SR. In other words, LTCC activity occurs during the second step in EC-coupling, directly after excitation and firing of the action potential in the sarcolemma. Increased LTCC activity is expected to increase the SR calcium load and/or calcium transient amplitude. Our results show a significant increase in LTCC protein levels for both Tm54 and Tm54/AE3 relative to the LTCC level in WT cardiac homogenates (Fig. 4.9). The increase in Tm54 shows a value of 137 ± 8% versus 136 ± 10% in Tm54/AE3 relative to WT levels. Surprisingly, the activity of LTCC in mutant cells, measured by whole cell patch-clamping, uncovers similar or even lower calcium current amplitudes versus WT cells (Fig. 4.9). This discrepancy between protein levels and LTCC activity may be due to post-translational modification of the channels which affects its activity [125, 126].
Figure 4.9. Immunoblot analysis revealed higher levels of LTCC in both Tm54 and Tm54/AE3 versus WT cardiac homogenates but similar calcium currents were found across the three genotypes. (A and B) Immunoblotting of total cardiac homogenates and the corresponding densitometry analysis. (C and D) Bar graph for calcium current density by using a whole-cell patch-clamping technique, along with the corresponding current/voltage (I/V) curve. N = 6 different hearts for each genotype in the immunoblot analysis. For calcium current analysis: n = 3 hearts (31 cells) for each genotype. *, p < 0.05.
**SERCA2a and PLN**

SERCA2a is the key protein responsible for sequestering calcium from the intracellular milieu into the SR, i.e., it is the mechanism for SR calcium loading. In contrast, phospholamban (PLN) is a negative regulator of SERCA2a activity in cardiomyocytes. Phosphorylation of PLN at two specific residues, Ser-16 and Thr-17, alleviates the inhibitory effects of PLN on SERCA2a activity.

SERCA2a and total PLN levels were similar across the three genotypes (Fig. 4.10). However, the level of phosphorylated PLN, both on Ser-16 and Thr-17, tends to be higher, albeit not significant, in Tm54/AE3 heart homogenates when compared with both WT and Tm54 levels. The level of p-Ser-16, a substrate for PKA, revealed an increase of $22 \pm 0.23\%$, whereas the level of p-Thr-17, a substrate of CaMKII, increase by $11 \pm 0.2\%$ (Fig. 4.10).
Figure 4.10. Levels of SERCA2 and total PLN are not changed across the three genotypes, however, a trend toward increased levels for p-PLN were observed for Tm54/AE3. (A) SERCA2a, (B) Total PLN, (C) PLN-PS16 and (D) PLN-PT17. S-actin and GAPDH are used as loading controls. (n = 6 hearts for each genotype).
RyR2 and NCX1 Levels

Next, we analyzed the level of RyR2, the SR calcium release channel. Similar levels were observed for the RyR2 across the three genotypes, with a slight reduction found in Tm54/AE3 double mutant heart homogenate (Fig. 4.11).

In contrast, an increase in NCX1 was observed for both Tm54 and Tm54/AE3 vs. WT levels, albeit being significant only in Tm54. Levels in Tm54 hearts showed an increase of 22%, whereas mean values for Tm54/AE3 hearts increased by only 10% (Fig. 4.11).

Figure 4.11. RyR2 levels are similar across the three genotypes but NCX1 levels in Tm54 heart homogenates are increased vs. WT. (A) Representative RyR2 immunoblot and the corresponding densitometry analysis. (B) NCX1 immunoblot and densitometry analysis. S-actin was used as loading control. (n = 6 hearts for each genotype). * p < 0.05 vs. WT level.
Levels of p-PLN and p-TnI in Isolated Cardiomyocytes After Pacing and ISO Stimulation

Finally, we performed experiments to determine what changes occur in phosphorylation of relevant proteins after electrical pacing or isoproterenol (ISO) treatment that might give rise to the divergent effects on calcium transients seen in Tm54/AE3 double mutant cardiomyocytes. Three key proteins relevant to calcium cycling were analyzed in this respect: phosphorylated phospholamban at Ser-16 (PLN-PS16), phosphorylated phospholamban at Thr-17 (PLN-PT17) and phosphorylated troponin I at Ser-23/24 (p-TnI). Our results indicate a robust increase in phosphorylation after pacing, especially for PLN-PS16 and PLN-PT17, and more prominently after ISO stimulation (Fig. 4.12). The increase is comparable between the three genotypes for the three proteins as analyzed by densitometry (data not shown).

In summary, analyzing phosphorylation levels for these proteins after pacing and ISO treatment did not provide evidence that changes in the phosphorylation status of these proteins were responsible for the observed increase in calcium transients in Tm54/AE3 cells versus WT and Tm54 cardiomyocytes.
Figure 4.12. Changes in phosphorylation levels are similar between the three genotypes in isolated cardiomyocytes after electric pacing (0.5 Hz) and stimulation with isoproterenol (ISO), 1μM). (A) Phosphorylated phospholamban at Ser 16; (B) Phosphorylated phospholamban at Thr 17; (C) Phosphorylated troponin I at Ser 23/24. GAPDH is used as loading control. N = 3 hearts for each genotype.
Discussion

Sodium is one of the principal ionic components in the cytosolic milieu of cardiac myocytes, which is regulated directly by multiple ion transporters, channels and pumps [2]. The major route for Na⁺ extrusion is via the Na⁺-K⁺-ATPase, whereas sodium influx is facilitated by different transporters including NHE1 and NCX1, among others. Other transporters that may contribute indirectly to sodium homeostasis in the cell are chloride-bicarbonate exchangers (AE1-3). This indirect effect on regulation of sodium levels is anticipated through a putative coupling relationship with sodium-independent acid extruders [3].

One of the major, if not the major, acid extruder in cardiomyocytes is NHE1 which mediates sodium loading through its activity of Na⁺ influx and H⁺ efflux across the plasma membrane. In contrast, anion exchanger isoform 3 (AE3) is considered to be one of the most important acid loaders in cardiomyocytes. Analysis of protein and mRNA levels revealed that AE3 is the most abundant anion transporter in heart when compared with other members of this family (AE1 and AE2) [11, 12, 14, 92, 122, 123]. AE3 activity, by extruding HCO₃⁻ and bringing in Cl⁻, would lead to acidification of the cytosol. Acidification by itself would be expected to be harmful to the cell physiologically. However, AE3 operating as an acid loader in concert with NHE1, as an acid extruder, would maintain intracellular pH (pHᵢ) at physiological levels. However, NHE1 activity will lead eventually to sodium loading. Sodium is one of the driving force components for NCX1, the sodium-calcium exchanger, and chemical gradient of sodium (as well as Ca^{2+}) across the sarcolemma will dictate the direction of NCX1 activity [2, 85]. When sodium is increased sufficiently on the intracellular side of the membrane, it will lead to
The increase in intracellular calcium in cardiomyocytes can be detrimental and lead to contracture injury. A major route for increasing intracellular $\text{Ca}^{2+}$ ($[\text{Ca}^{2+}]_i$) is through increases in intracellular $\text{Na}^+$ ($[\text{Na}^+]_i$), namely increasing NHE1 activity with subsequent increase in $[\text{Na}^+]_i$, which will trigger the influx of calcium through NCX1 [3]. In addition, an increase in sodium along with chloride, with the latter coming in via AE3, might trigger movement of $\text{H}_2\text{O}$ into the cells, via an osmotic effect, leading to an increase in cell volume and cardiac hypertrophy. So, keeping in mind the inherent coupling of NHE1 and AE3, it is conceivable that AE3 ablation might be beneficial in the context of contracture injury and cardiac hypertrophy.

There is substantial evidence for the beneficial effects of NHE1 inhibition on cardiac hypertrophy [67], post ischemia-reperfusion injury [55] and myocardial infarction [127]. However, the direct contribution of AE3 ablation on heart function has been addressed only recently [1]. In this last study AE3 ablation seems not to have detrimental or beneficial effects on heart function per se. However, when AE3 is ablated along with another sodium loading mechanism, namely the NKCC1 $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter, detrimental effects were observed, which may be due to depletion of sodium in subsarcolemmal spaces [1].

To test the hypothesis that AE3 ablation would be beneficial in the context of heart disease we crossed AE3 KO mice with a well described model of dilated cardiomyopathy (Tm54). It is interesting to note that this model has a defective myofilament, with low calcium sensitivity [82]. Given the putative contribution of AE3 in calcium homeostasis this is an important characteristic, which contrasts with the increased calcium sensitivity observed in the
Tm180 model [81]. Hence, it is a great opportunity to analyze the different roles, if any, of AE3 in the context of differences in myofilament calcium sensitivity. Surprisingly, our data showed an increase in calcium loading after AE3 ablation (with an increase in both calcium transient amplitude and SR Ca$^{2+}$ loading) in Tm54/AE3 double mutant cardiomyocytes but not in Tm54 cells. Importantly, there is no improvement in cardiac performance after AE3 ablation, with essentially similarly depressed heart function in Tm54 and Tm54/AE3 mice. Given the theory behind AE3 ablation and the putative effects on sodium loading, these results are bit of a surprise. On the other hand, reductions in H/BW ratio might suggest some useful effect on cardiac hypertrophy, although similar reductions in H/BW ratio were observed for AE3 KO versus WT mice, which suggest that this effect occurs irrespective of the pathology of cardiac hypertrophy.

Another puzzling observation which might counter the hypothesis that AE3 couples with NHE1 is that the latter is indeed increased, rather than reduced, but this increase occurred in both Tm54 and Tm54/AE3 hearts. In other words the increase in NHE1 is most likely due to the Tm54 mutation rather than AE3 ablation. However, this effect is not unexpected given the well documented buildup of cellular acidosis in cardiomyopathies [19]. Nevertheless, an interesting observation is the reduction in β–MHC levels in Tm54/AE3 versus Tm54 hearts. This might suggest a reduced level of cardiac stress after AE3 ablation. Given the consistent nature of the β–MHC data in tested hearts (6 mice), it would be interesting to monitor its long term effect. Survival analysis would be a plausible approach to dissect long term effects of AE3 ablation in Tm54 mice. It might turn out to facilitate a long-term cardioprotective effect.
In addition, cell mechanics data show that Tm54 and Tm54/AE3 cardiomyocytes are as efficient as WT cells for both contractility and relaxation. Hence, cell mechanics data suggest that defective contractility (especially at baseline) is likely attributed to cardiac remodeling or systemic changes rather than to changes in cardiomyocytes per se. Another question in this regard is why an increase in calcium transient amplitude in Tm54/AE3 ventricular cardiomyocytes is not reflected in a more efficient contractility vs. Tm54 and WT cells? One possible answer is that AE3 ablation might reduce calcium sensitivity further in Tm54/AE3 cells and the cells increase the calcium amplitude to maintain normal levels of cell contractility, especially in the context of the Tm54 cardiomyopathy (since AE3 KO cardiomyocytes have normal Ca$^{2+}$ transients vs. WT cardiomyocytes; data not shown). However, this view might be refuted by the fact that an increase in cardiomyocytes acidosis, which is likely not to be the case during AE3 ablation, reduces cardiac contractility via a decrease in calcium sensitivity [19]. More specific experiments are needed to elucidate the effect of AE3 ablation on pH$_i$ in the context of mutations, such as Tm54, that cause cardiomyopathy.

Nonetheless, the increase in calcium transient amplitude in Tm54/AE3 cardiomyocytes is quite puzzling, and may be an indication of an indirect role for AE3 in signal transduction and cross talk, with AE3 affecting some process acting between the myofilaments and effectors of calcium cycling. This hypothesis is supported by our contrasting findings between the effects of AE3 ablation in Tm54 and Tm180 mice (Chapter 3), in which the changes in calcium are in the opposite direction, with significantly lower calcium amplitude in Tm180/AE3 vs. Tm180 cardiomyocytes. In this case the Tm180 cardiomyocytes have higher calcium sensitivity, consistent with the possibility that AE3 ablation leads to lower calcium transient amplitudes in conjunction with a myofilament having higher calcium sensitivity (the case in Tm180), whereas
AE3 ablation facilitates a higher calcium amplitude in the case of lower calcium sensitivity (the case of Tm54). Obviously the story is much more complicated than this oversimplification, but analysis of calcium handling proteins may provide some clues for what is happening in the cells. For example, RyR2 expression is clearly reduced in Tm180 myocytes, which correlates with lower calcium transient amplitudes (Chapter 3). In contrast, RyR2 levels are not changed across the three genotypes in the Tm54 study described here. So, high calcium transient amplitudes in Tm54 cardiomyocytes, when compared with Tm180, may be partially attributable to the different levels of RyR2. Side by side analysis of Tm54 vs. Tm180 cardiomyocytes uncovers significantly high calcium transient amplitudes in Tm54 vs Tm180 (data not shown). However, this does not explain the higher levels of calcium transient amplitude in Tm54/AE3 versus Tm54. Inspecting the levels of phosphorylated phospholamban may explain partially the increased levels of calcium transient amplitude in Tm54/AE3. The double mutant cardiac homogenates tend to have higher levels of phosphorylated phospholamban than Tm54 single mutant hearts, i.e., the Tm54 animals have higher levels of SERCA2 inhibition with anticipated lower SR calcium load (as seen in caffeine-induced SR calcium release).

In addition, the LTCC protein levels are increased in both Tm54 and Tm54/AE3 versus WT cardiac homogenates even though the calcium transient is increased only in Tm54/AE3 cardiomyocytes. Paradoxically, the calcium current is similar or lower than that in WT cells for both Tm54 and Tm54/AE3 cardiomyocytes. So what is the basis for these discrepancies? It is possible that LTCC undergoes post-translational modifications affecting its activity and it is not simply the increase in protein levels that dictates a robust increase in calcium currents [125, 126]. Another possibility is that the increase in LTCC levels is in response to complicated alterations and remodeling effects at the whole organ level. Obviously this global effect is absent
in isolated cells due to the absence of needs encountered in vivo and eventually the cells tend to reduce the calcium current even though the protein is higher.

Overall, AE3 ablation displays very distinct effects in this model of DCM (with lower calcium sensitivity) versus the other model (Tm180) with HCM (and higher calcium sensitivity). It will be very interesting to investigate more closely the molecular events in these models in the context of AE3 ablation and calcium cycling.
Chapter 5: Angiotensin II infusion model of heart disease

Summary

Angiotensin II (AngII) treatment of mice was accomplished by subcutaneous implantation of Alzet minipumps, which released AngII in a constant rate of 1 ng/g/min for a duration of four weeks. AngII infusion induces robust cardiac hypertrophy in both WT and AE3 knockout (KO) mice. However, the increase in heart weight/body weight (HW/BW) ratio in mg/g of bodyweight is the same in both genotypes vs. control WT animals not receiving AngII (WT-AngII, 5.59 ± 0.12; AE3 KO-AngII, 5.73 ± 0.15; WT controls, 4.71 ± 0.07; AE3 KO controls, 4.70 ± 0.05; P < 0.001 for both AngII treated groups vs. WT and AE3 KO controls). Protein levels of AE3 were not significantly changed in WT-AngII versus WT controls not receiving AngII. Levels of fetal genes expression, β–myosin heavy chain (β–MHC) and atrial natriuretic peptide (ANP), were sharply upregulated in both WT-AngII and AE3 KO-AngII vs. WT controls. Analysis of heart function by transthoracic echocardiography showed essentially similar results for WT-AngII and AE3 KO-AngII mice during most of the 4 weeks of AngII infusion, with values not significantly different from baseline. An exception was in the first week after AngII infusion in which both WT-AngII and AE3KO-AngII animals showed significantly lower values for LVEDD and LVESD (left ventricular end diastolic or systolic diameter, respectively), but higher values for fractional shortening (FS%) and ejection fraction (EF%) versus baseline levels. In contrast, analysis of heart function by in vivo analysis of left ventricular pressure revealed significant reductions in LVP and contractility (dP/dt\text{40}) in AE3 KO-AngII vs. WT-AngII. The differences were observed at baseline and after β–adrenergic stimulation with dobutamine at 32 ng/g/min.
Introduction

Angiotensin II (AngII) is a small peptide (8 amino acids after processing), which is formed in vivo by the enzymatic action of angiotensin converting enzyme (ACE) on the precursor decapeptide angiotensin I cleaving two amino acids from the C-terminal side. AngII exerts its effect via the angiotensin II receptors, AT1 and AT2, which are G protein coupled receptors (GPCRs). The general consensus is that AngII exerts its action on cardiovascular function via AT1 specifically [128-131].

Several reports support the notion that AngII is involved in the development of cardiac hypertrophy partially through the upregulation of several genes that induce cardiac hypertrophy and fibrosis [132, 133]. In addition, AngII is reported to regulate cardiac contractility and protein synthesis in rats [134-136]. Supporting evidence is also accumulating for the hypertrophic effect of AngII in animal studies indicating that AngII antagonists lead to regression of cardiac hypertrophy [137, 138]. Moreover, AngII antagonists, angiotensin converting enzyme inhibitors (ACEs) and angiotensin receptor blockers (ARBs), are currently cornerstone drugs in the treatment and management of hypertension and its cardiovascular complications, indicating the key role of AngII in the development of heart disease [139, 140]. AngII contributes directly to development of high blood pressure via both its vasoconstrictive effects and its effects on renal function and the regulation of systemic fluid-volume homeostasis [141, 142]. Interestingly, previous studies show that anion exchangers (AEs, including AE3) are induced in cardiac myocytes by AngII treatment and that this induction is mediated via AT1 receptor [20, 94]. This makes it quite relevant to analyze AngII-induced heart disease in the context of AE3 ablation.
Therefore, in the present study we have used AngII as an induction agent to trigger cardiac hypertrophy, and have analyzed the effects of such induction on cardiac function in both WT and AE3 knockout (KO) mice.

**Results and Discussion**

In this project, we used a very different approach than that described in the earlier chapters. Rather than using transgenic mouse models of cardiomyopathies (as in Chapters 3 and 4), here we implanted minipumps (Alzet pump 1004) subcutaneously, which infuse specific doses of Angiotensin II (1 ng/g/min). Both WT and AE3 KO mice (on an FVB/N background) underwent this procedure and cardiac function was evaluated each week for a total of 4 weeks by transthoracic echocardiography. Final evaluation was accomplished by analyzing in vivo cardiac function using the closed-chest model. The overall working protocol for this project is depicted in Figure 5.1.

![Figure 5.1. The overall working protocol for experiments in this chapter.](image)
HW/BW Ratio and Development of Cardiac Hypertrophy

After 28 days of AngII infusion hearts were collected at the end of this protocol. Both WT-AngII and AE3 KO-AngII mice showed significant enlargement of their hearts when compared with WT animals not receiving AngII (Fig. 5.2). WT-AngII animals had a HW/BW ratio (in mg/g body weight) of 5.59 ± 0.12 whereas AE3 KO-AngII mice had a ratio of 5.73 ± 0.15 versus a value of 4.71 ± 0.07 for WT mice without AngII infusion (p <0.0001 for both genotypes vs. WT-No AngII). No significant difference was uncovered between WT-AngII and AE3 KO-AngII hearts ratio. This indicates that both WT-AngII and AE3 KO-AngII mice were reacting similarly to AngII infusion, at least with regard to cardiac hypertrophy.

Further confirmation of this observation was revealed by histology sections of these hearts showing similar levels of remodeling and enlargement (Fig. 5.2). In addition, the level of fibrosis was also similar for AE3KO-AngII and WT-AngII as shown in the histology sections that were stained with Masson’s Trichrome to uncover the level of fibrosis (Fig. 5.2).
Figure 5.2. Cardiac hypertrophy occurred in both WT and AE3 KO mice receiving infusions of Angiotensin II. (A) HW/BW ratio obtained by dividing the heart weight (in mg) by the body weight (in g). (B) & (C) Longitudinal and cross sections of the hearts stained with Masson’s Trichrome for both WT-AngII and AE3 KO-AngII as indicated. All of the animals are 3 month old males (for HW/BW ratio, n = 8 for each genotype).

Levels of AE3, β-MHC and ANP

Previous reports suggested that AE3 activity is induced by AngII [20] and since our focus in this project is to analyze the effect of AE3 ablation on AngII-induced heart disease it is very much relevant to analyze the level of AE3 after AngII treatment. We analyzed the levels of AE3 in cardiac homogenates from WT animals receiving AngII and compared it with the levels in cardiac homogenates from WT mice not receiving AngII. AE3 levels were increased, albeit not significantly, after AngII infusion compared with mice not receiving AngII (Fig. 5.3). Densitometry analysis show that AE3 levels were upregulated by ~25% in WT-AngII vs. WT mice that were not treated with AngII (p = 0.1) (Fig. 5.3).
In addition, levels of β–myosin heavy chain (β–MHC) and atrial natriuretic peptide (ANP), both encoded by fetal genes known to be upregulated in the heart under stress conditions, were increased sharply for both WT-AngII and AE3 KO-AngII versus WT animals not receiving AngII. No differences were observed between WT-AngII and AE3 KO-AngII hearts. These data are consistent with the observations on HW/BW ratios and histology sections, and suggest that both WT and AE3 KO hearts are stressed similarly by AngII infusion.

Figure 5.3. AE3, β–MHC and ANP are increased in mice receiving AngII. (A) Immunoblot and densitometry analysis of AE3 in WT-AngII vs. WT heart homogenates. (B) β–MHC immunoblot and densitometry analysis in WT-AngII vs. AE3 KO-AngII (WT without AngII is not included). (C) Immunoblot and densitometry analysis for ANP in WT not receiving AngII, WT-AngII and AE3 KO-AngII. GAPDH serves as loading control. (n = 3-6 hearts per genotype). *p < 0.05.
Echocardiography Data

The overall data from echocardiography showed no differences between WT-AngII and AE3 KO-AngII throughout the entire 4-weeks protocol. The results showed that both groups had similar structural (LVEDD and LVESD) and functional (FS% and EF%) parameters, both at baseline and all the way through the succeeding 4 weeks of AngII infusion (Fig. 5.4). The results are unexpected for both genotypes since there is not much difference from baseline after AngII infusion. The most dramatic changes were observed in the first week after starting AngII infusion. The LVEDD and LVESD dropped significantly at this time point, which might reflect a rapid development of cardiac hypertrophy; however, the values returned back to near baseline levels in the following 3 weeks. It might be an adaptation of the mice to the chronic infusion of this large dose of AngII. However, how mice manage to “dissipate” the effect of AngII is not clear, but genetic background might be a major factor in this observation.

The functional parameters (FS% and EF%) showed generally the same profile including a return back to the baseline level starting from the second week of AngII infusion (Fig. 5.4). However, unexpectedly, after the first week of AngII infusion all of the mice (WT-AngII and AE3 KO-AngII) showed an increase, rather than a reduction, in FS% and EF% which are significant relative to baseline level. Why is this? Again there is not a clear-cut explanation, but the first week of infusion of AngII, which is a natural inotropic agent, might reflect this positive effect before receding to the pathological effects of chronic AngII infusion. However, after the first week the mice did not display a compromised FS% and EF%. Rather, they retained essentially similar levels as that found at baseline for the remaining 3 weeks.
An important issue to explain some of the unexpected observations here is that echocardiography as a technique is not as sensitive as, for example, the in vivo analysis of cardiac function via a catheter in the left ventricle, and it will detect only overt remodeling and functional changes. This can be well appreciated by examining the in vivo analysis of heart function at the end of the 4th week of AngII infusion (see below).

Figure 5.4. Echocardiography shows similar structural and functional changes in WT-AngII and AE3 KO-AngII mice. (A) Left ventricular end-diastolic dimension (LVEDD), (B) Left ventricular end-systolic dimension (LVESD), (C) Percent of fractional shortening (FS%), (D) Percent of ejection fraction (EF%) (n = 8 mice for each genotype).
In vivo Analysis of Heart Function

The in vivo analysis of left ventricular performance, was conducted at the end of the echocardiography protocol, and after 4 weeks of subcutaneous infusion of AngII. The data showed some significant differences between the two genotypes either at baseline or after β–adrenergic stimulation with a single dose of dobutamine at 32 ng/g/min (Fig. 5.5).

Generally, the AE3 KO-AngII mice exhibited lower values for blood pressure parameters (MAP, LVP and systolic pressure). For example LVP was significantly reduced in AE3 KO-AngII vs. WT-AngII at baseline and after β–adrenergic stimulation (Fig. 5.5). Previously, the same observation with regard to blood pressure was made in the Tm180/AE3 double mutant vs. Tm180 single mutant (Chapter 3) but not in Tm54/AE3 double vs. Tm54 single mutants (Chapter 4). This likely related to the model type since both the experimental approach in this chapter, using AngII treatment, and the Tm180 mutation produce cardiac hypertrophy as a common remodeling effect. In contrast, the Tm54 mutant is different in that they develop cardiac dilatation rather than cardiac hypertrophy as the primary remodeling effect.

Interestingly, we found that contractility, especially after normalizing for differences in afterload (dP/dt₄₀), is significantly reduced in AE3 KO-AngII vs. WT-AngII mice (Fig. 5.5). This indicates that AngII treatment has more severe functional consequences in AE3 KO mice than in WT mice. This difference was not found by echocardiography, likely because of the less sensitive nature of echocardiography when compared with the analysis of left ventricular performance using a pressure transducer in the left ventricle. Again these data are in agreement with the observations using the Tm180 model, in which the Tm180/AE3 double mutant has more severely compromised heart function when compared with the TM180 single mutant (Chapter 3).
Relaxation, as indicated by \(-dP/dt\), was also reduced, although not significantly, in AE3 KO-AngII vs. WT-AngII.

**Figure 5.5.** In vivo analysis (baseline and 32 ng dobutamine) of mice undergoing AngII infusion for 28 days showed more prominent depression of heart function in AE3KO-AngII vs. WT-AngII mice. (A) Heart rate, (B) Mean arterial pressure, (C) Left ventricular pressure, (D) Cardiac contractility, (E) Cardiac relaxation, (F) Normalized contractility at 40 mm Hg afterload. (n = 7 mice for each genotype with same age and gender) * P < 0.05.
Angiotensin II Infusion and Blood Pressure Development

Due to the observations using echocardiography and subsequent finding in analyses of left ventricular performance, we conducted a pilot study using two mice to monitor development of high blood pressure after AngII infusion. Both of the mice were WT and underwent telemetry probe implantation for direct and continuous measurements of arterial blood pressure in conscious mice. The same two mice received an Alzet minipump for the continuous infusion of AngII with the same dose as used for the earlier study (1 ng/g/min).

The data show that blood pressure is steadily increased during the 5-day recording protocol from a baseline of 100 mm Hg to 148 mm Hg at day 5 after beginning infusion of AngII (Fig. 5.6). These data confirm that AngII is indeed exerting an effect on blood pressure in mice undergoing AngII infusion. However, we made another observation, which is relevant to the analyses of left ventricular performance using a pressure transducer. When the anesthesia agents were injected into these mice, first Ketamine and then Inactin, a dramatic reduction in blood pressure occurred (Fig. 5.6). Since Ketamine and Inactin are used for closed chest assay but not echocardiography, this observation is relevant to the experiments in Fig. 5.5. This might explain the lower blood pressure parameters recorded in mice in those studies (see above). However, these observations apply to both WT and AE3 KO as seen in the studies of left ventricular performance. It is possible that this interaction is related to the genetic background of these mice since other mice with different genetic background did not show such interactions (data not shown).
Figure 5.6. Angiotensin II infusion (1 ng/g/min) causes a steady increase in blood pressure as measured by telemetry probe implantation. The blood pressure dropped dramatically after injection of ketamine and inactin injection. Each line represents one mouse.

Conclusion

Overall, the data shown in this chapter indicate that AngII infusion causes robust cardiac hypertrophy without significant differences between AE3 KO-AngII and WT-AngII mice. Functional differences can be recognized with more severe presentation in AE3 KO-AngII vs. WT-AngII by using more sensitive techniques for evaluating heart function.
Chapter 6: Conclusions and Future Directions

The work presented in this thesis is concerned with the cardiovascular functions of the AE3 Cl⁻/HCO₃⁻ exchanger and the effects of its activity under conditions of heart disease. AE3 is highly expressed in excitable tissues, namely in the brain and heart, with negligible levels in most other tissues [12, 14, 122]. Given the high levels of AE3 expression in excitable tissues, it is important to understand the function of AE3. One of the most useful means of obtaining information that can contribute to elucidation of the in vivo functions of a given protein is to generate and analyze a mouse model in which its gene has been disrupted. Global ablation of AE3 was reported to have negative effects in neuronal tissues, which exhibited a lower threshold for agent-induced seizures [22]. This effect is reasonable given the high levels of AE3 expression in neuronal tissues and the potential for Cl⁻/HCO₃⁻ exchange to affect Ca²⁺ handling.

In contrast, mice lacking AE3, unexpectedly, exhibit normal cardiac function, with efficient contractility and relaxation both under baseline conditions and after β-adrenergic stimulation [1]. These results were puzzling given the high levels of expression of AE3 in heart, and the existence of a cardiac-specific splice variant [12]. It seems unlikely that a cardiac-specific variant of AE3 would be expressed at such high levels in heart, but not serve an important function. Nonetheless, since at least three other Cl⁻/HCO₃⁻ exchangers are expressed in the heart (AE1, AE2 and PAT1) [11, 12, 14, 92, 122, 123], the absence of an effect on cardiac function in AE3-null mice might be attributed to this redundancy and functional compensation from other exchangers. However, ongoing work on the PAT1 KO in our lab indicates that this Cl⁻/HCO₃⁻ and Cl⁻/OH⁻ exchanger probably has a distinct function compared with AE3 (Prasad, V.; personal communication). PAT1 KO mice indeed have better contractility than WT mice.
after β-adrenergic stimulation. Nonetheless, more detailed analyses of AE3 and its role in cardiac function shows that AE3-null mice exhibit a significant reduction in force-frequency response (FFR) when compared with WT mice (Prasad, V; manuscript in preparation). The positive force-frequency response is a major mechanism by which the heart responds to higher demands for cardiac output. In contrast, a negative or blunted force frequency response is a major hallmark of failing heart and cardiac decompensation [124].

A major reason that we initiated the investigations in this thesis was that a number of investigators suggested that AE3 inhibition might serve as a potentially promising therapeutic strategy for treatment of heart disease. Indeed, pharmacological inhibition of Cl⁻/HCO₃⁻ exchange by relatively non-specific stilbene inhibitors was found to protect against cardiac arrhythmias associated with ischemia and reperfusion injury [4]. This effect was attributed, partially, to alteration in [Cl⁻]ᵢ which is maintained at levels above its equilibrium potential in normal cardiac muscle due to the activity of AE3 and/or other Cl⁻/HCO₃⁻ exchangers [4].

In addition, a conceptual belief that AE3 ablation would be beneficial and cardioprotective stems from the postulated coupling relationship between AE3 and NHE1. NHE1 is accepted as one of several sodium loading mechanisms and its inhibition, either pharmacologically or genetically, proved to be therapeutically beneficial vis a vis cardiac hypertrophy and ischemia-reperfusion injury, at least in animal models [3, 55]. This effect is attributed, at least partially, to the reduction of [Na⁺]ᵢ with subsequent effects on NCX1 activity. Low levels of [Na⁺]ᵢ are expected to increase the driving force for calcium efflux via NCX1 leading to a reduction in both [Ca²⁺]ᵢ and its damaging effect in cardiomyocytes under stress.
conditions. Therefore, inhibition of AE3 activity was expected to be useful therapeutically because of its inhibitory effects on NHE1 activity.

The findings presented in this thesis argue against this hypothesis, at least for the models used for these investigations. It turned out that AE3 ablation has no effect on cardiac hypertrophy in two of the mouse models analyzed (the Tm180 hypertrophic cardiomyopathy model and mice subjected to AngII infusion), and only a modest reduction was observed in the third model (the Tm54 dilated cardiomyopathy model). More dramatically, AE3 ablation indeed leads to worsening of heart disease in the TM180 model (Chapter 3). Tm180/AE3 double mutant mice exhibited much more severe heart disease and died prematurely due to heart failure. Negative effects of AE3 ablation were also seen in the AngII infusion model when heart function was analyzed using a more sensitive procedure than echocardiography (Chapter 5). Indeed, both of these models (Tm180 and AngII infusion) are primarily cardiac hypertrophy models, whereas Tm54 is primarily a dilatation model. It is interesting to note that AE3 ablation in Tm54 mice did not lead to worsening of the disease condition, as observed for the TM180 model. This might indicate different effects of AE3 ablation depending on the model being used and the relevant underlying pathophysiology.

A major question now is whether it is possible that AE3 inhibition might have beneficial effects in human patients with heart disease? From the findings presented in this thesis, it is highly unlikely that it would have positive effects, especially in similar disease conditions. However, the possibility remains that such a positive effect is attainable simply due to the fact that large differences do exist between human and small animal models such as rats and mice. In other words the mouse model might not accurately represent the human version of these
diseases. For instance, one might argue that sarcolemmal calcium efflux due to the contribution of NCX1 in human cardiomyocytes is 30%, in contrast to barely 10% in rats and mice, with the remaining 70% and 90% attributed to SERCA2. In addition, species other than mouse and rat, including human, have intracellular $[Na^+]_i$ in the range 4-8 mM, whereas it is 10-15 mM in rat and mouse cardiac myocytes [2]. Also differences do exist between rat and human cells in NHE1 activity, the exchanger presumed to function in concert with AE3. For example, the rate of acid efflux via NHE1 in rat is 2.8 mM/min, with concomitant Na$^+$ loading, versus a rate of 1.1 mM/min in human cardiomyocytes. Taken together this might limit Ca$^{2+}$ extrusion in rat and mouse during diastole versus human cardiomyocytes with obvious ramifications on Ca$^{2+}$ amplitude and kinetics. Most importantly this might contribute to distinct differences in cardiac injury and decompensation between mice and rats versus other species, including human. In summary, NCX1 activity and steady state $[Na^+]_i$ are distinctly different between mouse and rat versus other species. Therefore these are valid concerns and might be addressed by analyzing the effects of AE3 inhibition in large animal models of heart disease (e.g., rabbits, dogs, or pigs). For such studies it would be necessary to develop specific inhibitors for AE3.

An interesting observation in this research is that Tm180/AE3 double mutants and Tm180 single mutants have a more severe disease phenotype versus Tm54/AE3 and Tm54 mutants, despite the less severe depression of cardiac function in the TM180 mutants under basal conditions. That is, the Tm54/AE3 and Tm54 mutants show apparently a more severe deficit in cardiac performance especially when we are analyzing cardiac contractility and relaxation by intraventricular pressure measurements. Tm54/AE3 and Tm54 mutants have significantly reduced contractility at baseline versus WT mice, whereas in Tm180/AE3 and Tm180 the baseline contractility is same as in WT animals. In regard to heart function under conditions of
β–adrenergic stimulation, Tm54/AE3 and Tm54 mice showed a reasonable increase in contractility, which reached its highest value at 32 ng dobutamine. The largest value attained for contractility in Tm54/AE3 and Tm54 mice no higher than the baseline values for Tm180/AE3 and TM180 mutants. However Tm180 mice, and especially Tm180/AE3 mice, had a blunted response to β–adrenergic stimulation. Our prediction is that the level of cardiac stiffness is a major contributor to the increased severity of the disease but we know that PLN-phosphorylation is largely maxed out at baseline so this explains the blunted β–adrenergic response in Tm180/AE3 and Tm180. The stiffness attributed to enlarged and heavily fibrotic atria is obvious in all histology sections of Tm180/AE3 and Tm180 mice which does not occur in the Tm54/AE3 and Tm54 mutants. On the other hand, the reduction in contractility under baseline conditions in the Tm54/AE3 and Tm54 mice may be due, at least in part, to the prominent dilatation and its negative effects on contractility and relaxation. Also, reduced baseline contractility in Tm54/AE3 and Tm54 mutants can be attributed to lower calcium sensitivity of the myofibrils versus Tm180/AE3 and Tm180 mutants. Indeed, there is large difference between Tm54 and Tm180 in calcium sensitivity, with WT calcium sensitivity in the middle point between them [81, 82].

Another observation worth noting is the differential effects of AE3 ablation on calcium handling, depending on the animal model being used, which may be related to the level of calcium sensitivity. For example the Tm180 model is a hypertrophic cardiomyopathy model and is characterized by high myofibrillar calcium sensitivity [81, 82]. When calcium was analyzed in Tm180 cardiomyocytes in the context of AE3 ablation (Tm180/AE3 double mutant) there was a highly significant reduction in the calcium transient amplitude versus Tm180 and WT mice. In
contrast, in Tm54 mice, which is a dilated cardiomyopathy model with lower calcium sensitivity [81, 82], the ablation of AE3 (Tm54/AE3 double mutant) leads to a highly significant increase in calcium transient amplitude versus Tm54 and WT cardiomyocytes. Interestingly, the level of the calcium transient amplitude is not changed in Tm54 cardiomyocytes compared with that of WT cells, whereas the level in Tm180 cardiomyocytes is significantly lower than in WT cells. It is intriguing that AE3 ablation leads to opposite effects on calcium transient amplitude in the context of two different α–tropomyosin mutations (Tm54 vs. Tm180) and/or changes in myofibrillar calcium sensitivity. Is it an indication of cross-talk between AE3 and myofibrillar proteins, at least indirectly through signaling proteins? Or are these effects due to distinct alterations in intracellular ionic milieu and pH\textsubscript{i} in the bulk cytosol or in microdomains, such as subsarcolemmal spaces? These are open and valid questions and warrant further investigation. Nonetheless, analyzing variations in [Na\textsuperscript{+}]\textsubscript{i} and pH\textsubscript{i} of the bulk cytosol in the context of AE3 ablation will be an important set of experiments to begin deciphering the mechanisms underlying the observed alterations in calcium handling.

In conclusion, AE3 ablation is unlikely to be a favorable therapeutic target for cardiac hypertrophy and other forms of heart disease. However, some interesting results, especially in Ca\textsuperscript{2+} cycling, warrant further work to elucidate the underlying molecular alterations due to AE3 ablation.
REFERENCES


