DEFINING A ROLE FOR THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE
REGULATOR IN THE HEART

BY

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DISSERTATION

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ABSTRACT

While cystic fibrosis (CF) is commonly thought of as a lung disease, since it's first

description 70 years ago we have come to understand that loss of CFTR (cystic fibrosis

transmembrane conductance regulator) function affects numerous tissues and systems throughout

the body. Concerning the heart, right ventricular dysfunction secondary to pulmonary

abnormalities has long been recognized. However, the issue as to whether loss of CFTR

function causes primary disturbances in the heart has remained an unsolved and often debated

question. Thus, the goal of the research presented herein was to answer two fundamental

questions, 1) "Does CFTR play a physiological role in heart function?," and 2) "What direct

impact does loss of CFTR activity have on heart function?" These are particularly timely

questions now as CF patients are living longer and the development of CFTR modulators is

being pursued with increased vigor. Thus, an understanding if CF patients will be at increased

risk for heart disease and whether new therapies may affect heart function are of direct relevance

to the care of these individuals.

To begin to answer the questions presented we have examined how modulation of CFTR

affects neonatal ventricular cardiomyocyte contraction rate. We found that CFTR is involved in

the regulation of both baseline and β-adrenergic stimulated contraction rate. Additionally, loss

of CFTR activity, whether acutely or chronically, leads to modulation of intracellular signaling

molecules and other Cl⁻ channels, which in some cases can completely compensate for this loss,

while in other cases cannot. Finally, *in vivo* examination of heart function in CFTR KO mice has

elucidated disturbances in heart structure and function, which may translate into problems

experienced by CF patients. In sum, this work presents a significant expansion of our

understanding regarding the role of CFTR in heart function and highlights the need to further

pursue a more in-depth analysis of the health of the heart in CF patients.

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CHAPTER 1:

PROPERTIES OF THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR AND ITS POTENTIAL RELEVANCE TO CARDIAC CONTRACTION RATE

Cardiovascular disease is the number one killer in the United States. Statistics taken in 2004 by the American Heart Association show that over 74 million people are affected by cardiovascular diseases, with an economic impact over 430 billion dollars. Diseases specifically affecting the heart, namely heart disease, stroke, and sudden cardiac death, account for a significant proportion of that impact (www.americanheart.org). With a risk of sudden cardiac death six to nine times that of the general population, at least 50% of deaths in patients with heart failure are due to ventricular arrhythmias (1, 2). Therefore, understanding the physiological and pathophysiological processes in the heart remains an important area of investigation.

CARDIOMYOCYTE CONTRACTION RATE AND THE ROLE OF ION CHANNELS IN RATE MODULATION

Cardiac muscle, like skeletal muscle, is a striated muscle and thus the two muscle types share many characteristics. However, the electrophysiology of the two muscle types differs dramatically. Unlike skeletal muscle, the cardiac action potential is not initiated by neural activity, but rather modulated by it. The heart contains two broad types of cells: 1) contractile and 2) conductile. The contractile cells are those within the atria and ventricle responsible for the contraction of the myocardium. Whereas, the conductile cells are specialized muscle cells involved in the initiation and propagation of action potentials and typically have little mechanical

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capability. These cells generally make up the sinoatrial (SA) and atrioventricular (AV) nodes along with the bundle of His and Purkinje cells. In the normal human heart, each heartbeat is driven by the primary pacemaker cells of the SA node, which initiates and then propagates the action potential across the atria, resulting in contraction of the atria. Additionally, the AV nodal cells are activated and serve to spread the action potential to the ventricles via the bundle of His and Purkinje fibers. While in healthy hearts, SA nodal cells are responsible for the automaticity of the heartbeat; all heart cells have the capability to generate a spontaneous action potential. Under normal conditions, SA nodal cells drive the heartbeat because they generate ~80-100 action potentials/minute, which overrides the AV nodal cells and bundle of His with ~40-60 and 15-30 action potentials/minute, respectively (3). However, if the function of the SA nodal cells is lost, AV nodal cells or those of the bundle of His can keep the heart beating, although this results in the atria and ventricles beating independently of one another. Under this condition, called complete heart block, usually the reduced ventricular rate is too slow to provide sufficient cardiac output for the body. Additionally, while contractile cells typically do not have pacemaker activity, they can be induced (e.g. during ischemia) into spontaneous depolarizations, resulting in ectopic heartbeats. Furthermore, neonatal ventricular cardiomyocytes, which are often used in research due to their plasticity, resemble a hybrid between conductile and contractile cells. When cultured to form a syncytium they spontaneous beat similar to SA nodal cells. Research has shown that these cells contain all of the primary ion channels needed for spontaneous activity and contraction, including the hyperpolarization-activated 'funny' channel (I_f), T-type and L-type Ca^{2+} channels, inward Na^+ channels, and inward and outward K^+ channels (4).

The roles of cationic currents (Na^+, Ca^{2+}, K^+) in setting cardiac action potentials and in contraction rate have been well studied. Figure 1.1 illustrates the individual roles of these cationic currents in generating cardiac action potentials. In spontaneously beating rat ventricular myocytes, I_f has been shown to be critical in the generation of action potentials (5). However, using SA nodal rabbit myocytes, Vinogradova *et al* found that high basal PKA activity and rhythmic oscillations in Ca^{2+} release from sarcroplasmic reticular stores contributed significantly to spontaneous beating (6). Therefore, we see that spontaneous contraction rate is controlled by multiple processes involving ion channels, kinases, and Ca^{2+}-signaling proteins.

While cationic currents are typically seen as the "drivers" of contraction rate, anion currents (notably Cl^-) have been less studied due to their perception as purely "modulators." Despite their dwarfed status, roles for Cl^- channels in action potentials have been investigated. Over the past 10 years, several extensive reviews examining the expression and function of Cl^- channels in the heart have been published (7-16). Similar to other cells, in cardiac myocytes, Cl^- channels are expressed in the sarcolemmal membrane, sarcoplasmic reticular membrane, mitochondrial membranes, and nuclear membranes (17). In the heart, most Cl^- channels fall into four types, which consist of the outward-conducting Cl^- channels (swelling-activated Cl^- channels, Ca^{2+}-activated Cl^- channels, and the cystic fibrosis transmembrane conductance regulator), and inward rectifying Cl^- channels. As inward-rectifying Cl^- channels have significantly distinct roles from outward-conducting Cl^- channels, they will not be discussed here, rather we will focus our discussion on those channels which have potential functions most resembling CFTR.

NON-CFTR OUTWARDLY CONDUCTING CARDIAC Cl⁻ CHANNELS

Swelling-activated Cl⁻ channels are important in maintaining the intracellular osmolarity and volume in response to changing extracellular environments. While the molecular identity of this channel is controversial, its biophysical and regulatory characteristics are well understood. Similar to other cell types, in ventricular cardiomyocytes swelling-activated Cl⁻ currents display moderate outward rectification, lack time-dependent activation upon depolarization, and are inhibited by tamoxifen, NPPB, and DIDS (18). Activation of swelling-activated Cl⁻ current is stimulated by changes in cell volume, osmolarity, membrane tension, and direct mechanical stretch. Activation depends on the presence of intracellular ATP; however, no ATP hydrolysis is necessary (19, 20). It has been suggested that the dependence on cytosolic ATP may serve to prevent the loss of metabolically valuable intracellular organic osmolytes during starvation (19, 21). Additionally, the activation of swelling-activated Cl⁻ currents is modulated by the intracellular Cl⁻ concentration, as high $[Cl^-]_i$ shifted the set-point of activation to larger volumes or decreased the rate of activation (22-24). Because it is activated by mechanical stress, swelling-activated current is considered a potential effector of mechanoelectrical feedback. Swelling-activated current is activated in ischemic and non-ischemic dilated cardiomyopathies and plays a role in arrhythmogenesis, myocardial injury, preconditioning, and apoptosis of myocytes (8).

Electrophysiological analysis of Ca^{2+}-activated currents in numerous cell types has shown that widely different single-channel conductances exist, ranging from 1 to 70 pS, suggesting a molecular diversity of Ca^{2+}-activated Cl⁻ channels (CaCC). In ventricular myocytes, small 1 pS Ca^{2+}-activated Cl⁻ currents that are sensitive to niflumic acid and DIDS have been recorded (25, 26). While these currents are typically outwardly rectifying and show time-dependent activation upon depolarization, as cytosolic Ca^{2+} concentrations increase, both

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characteristics gradually decrease to almost time-independent activation and a linear *I-V* curve

(27). As the name suggests, Ca^{2+}-activated currents are activated by increases in intracellular

Ca^{2+} (Ca^{2+}_i), resulting from Ca^{2+} influx via voltage-gated Ca^{2+} channels and/or release of Ca^{2+}

stores from the sarcoplasmic reticulum. Additionally, CaCC are activated by phosphorylation

from Ca^{2+}-dependent kinases such as calmodulin-dependent kinase II (CaMKII) (28). The

importance of CaCC in appropriate action potential formation has been well described. CaCC

has been shown to be responsible for the early repolarization phase I_{to2}, which is 4-

aminopyridine-insensitive (28). This was illustrated by Xu *et al*, who found that inhibition of

CaCC by niflumic acid led to prolongation of the early repolarization of the cardiac action

potential (26).

In summary, it has been thought that outwardly conducting Cl^- channels share a similar

role in maintaining the resting membrane potential and minimizing action potential prolongation

associated with stimulation of inward Ca^{2+} current. As a result, these Cl^- channels may be

arrythmogenic and prevent hypercontraction through prevention of Ca^{2+}_i overload (10, 25, 29)

(Figure 1.2).

STRUCTURE AND ELECTROPHYSIOLOGICAL PROPERTIES OF CFTR

Structure. CFTR is a member of the family of ATP-binding cassette (ABC)

transporters, the largest family of known ion channels. Like other members of this family, CFTR

is a 12 transmembrane protein with 2 membrane-spanning domains, each consisting of 6

transmembrane motifs, with cytosolic N- and C-termini. While no crystal structure for CFTR

exists, based on amino acid sequence, hydropathy plots, and crystallization of related

components (30), models of CFTR have been generated **(**Figure 1.3). Extracellularly, CFTR is

heavily glycosylated. Experiments examining CFTR variants lacking the consenses

glycosylation sequences in the extracellular loop suggest that glycosylation is not necessary for

correct protein processing or for the formation of a phosphorylation-regulated Cl$^-$ channel (31,

32). However, anion efflux by these mutations did alter the time course of channel activity,

suggesting glycosylation may be involved in channel function (31). The role of glycosylation in

the maturation and function of CFTR and its relation to clinical disease remains an active area of

investigation (33). Intracellularly, CFTR consists of two nucleotide-binding domains (NBD) and

a regulatory (R) domain. The roles of these domains in CFTR physiology and pathophysiology

are an area of intense investigation and are thoroughly reviewed elsewhere (34-36). Briefly, like

other ABC transporters, the NBDs of CFTR are responsible for ATP hydrolysis. This hydrolysis

by both NBDs controls both the opening and closing of CFTR channels. However, ATP

hydrolysis of NBDs is dependent upon phosphorylation of the R domain, which contains several

phosphorylation sites. Although CFTR is classically described as a "cAMP-stimulated" channel

due to activation of CFTR by PKA, the R domain of CFTR contains multiple phosphorylation

sites, which regulate its channel activity. While cAMP/PKA-activation is generally believed to

be the major form of activation, CFTR is also activated by PKC (in the presence and absence of

Ca^{2+}) (37) and cGMP/PKG (38). In addition to these kinases, phosphorylation of the R domain

is regulated by serine/threonine protein phosphatases, most notably PP2A (37). The importance

of NBDs in channel activity and the presence of multiple disease causing mutations within these

regions has spurred a significant effort to understand the ways in which these NBDs affect CFTR

channel function. Additionally, as our understanding regarding the complex signaling pathways

found in cells expands, so does our knowledge about the role of CFTR and its R domain.

Electrophysiological properties. Unlike many of the other ion channels found in the heart, CFTR is a voltage-independent channel. Under symmetrical Cl^- conditions, CFTR channels exhibit a linear *I-V* relationship, although in asymmetrical conditions, this can rectify. CFTR has been shown to have a low single-channel conductance of 6-10 pS (39, 40). Reversal potential measurements with different NaCl gradients indicate that CFTR is selective for anions over cations. In whole cell patch, the anion permeability of CFTR is $Br^-{\geq}Cl^->I^->F^-$ (41). However, in excised patches, the permeability sequence changes to $I^->Br^->Cl^->F^-$. This discrepancy was explained by observations that I^-, unlike other halides, blocks the pore of CFTR, thus rendering I^- less permeable under whole cell conditions (42). Additionally, CFTR is permeable to some polyatomic ions, such as NO_3^-, HCO_3^-, formate, and acetate (in that order of permeability), but not others, including pyruvate, propanoate, methanesulfonate, ethanesulfonate, and gluconate (43). CFTR has also been shown to be permeable to ATP (44) and glutathione (45), although the physiological significance of this is still under investigation. While this data gives insights into the pore of the channel, it is well accepted that physiologically CFTR functions as an anion channel that conducts both Cl^- and HCO_3^-.

TISSUE DISTRIBUTION OF CFTR EXPRESSION

CFTR is broadly expressed throughout the body, including both epithelial and non-epithelial tissues. By far, CFTR has been most widely examined in epithelial tissues, where morbidity and mortality affecting cystic fibrosis patients, whom have decreased to no CFTR activity or expression, has classically most significantly affected epithelial tissues. As a sampling, in humans, CFTR is expressed in the sweat glands, along the respiratory tract, gastrointestinal tract, and reproductive tissues. Although less studied, it has also been

documented that CFTR is expressed in other tissue types, such as immune cells (46), bone (47), nervous tissue (48, 49), and muscle (smooth (50), skeletal (51), and cardiac). In the heart, CFTR expression is well accepted in several animals, including rats (52), mice (53), guinea pigs (54), rabbits (55), swine (56), dogs (54), and primates (57). In general, CFTR is predominantly expressed in ventricular myocytes, although it has been reported in atrial cells (57). The story regarding CFTR expression in humans has received much debate, as conflicting reports exist. While Warth *et al* reported expression and activity of CFTR in human atrial myocytes (57); Berul *et al* found none (58). In contrast, in human ventricular myocytes, multiple groups have found both expression and activity of CFTR, although it does appear to be expressed at levels significantly lower than epithelial cells (57, 59).

CFTR FUNCTION

By far CFTR is most widely regarded as a Cl^- channel. In all tissues examined, CFTR has been shown to be an important ion channel in the homeostatic regulation of salts. Depending on the tissue type, this may involve absorption of salts (e.g. sweat glands), while in others it involves secretion (e.g. intestines). In cardiac myocytes, ions can move in either direction depending on the membrane potential. Similar to swelling-activated and Ca^{2+}-activated Cl^- channels, it is has been postulated that CFTR serves as a regulator of cardiomyocyte membrane potential and action potential duration. In recent simulation studies it was found that under β-adrenergic stimulated conditions CFTR density altered the action potential duration along with the membrane potential (60) (Figure 1.4). However, whether altered CFTR density or activity affects heart function experimentally has yet to be shown.

As mentioned previously, CFTR is not just a Cl^- channel, but is also permeable to other anions, most notably HCO_3^-. Beyond its role in salt regulation, CFTR also has a confirmed role in the regulation of pH. CFTR can modulate extracellular and intracellular pH directly through its conductance of HCO_3^- (61, 62) or indirectly through its interactions (functional and/or physical) with Cl^-/HCO_3^- exchangers (63, 64), Na^+/H^+ exchangers (65), carbonic anhydrase (66), and intacellular V-ATPases (46). This, together with the potential roles of CFTR in ATP signaling (44) and redox regulation (45), highlights the importance of CFTR in physiological functions.

CFTR AND CYSTIC FIBROSIS

Cystic fibrosis (CF), with mutations in the gene coding for the anion conductance channel CFTR, epitomizes a disease whose clinical manifestations are due to defective ion transport. As the most common lethal genetic disorder among Caucasians, CF affects nearly 30,000 individuals in the U.S. per year (www.cff.org). Since the discovery that the primary defect in CF involves mutations in the gene encoding for CFTR, extensive research efforts have been dedicated to characterizing the role of CFTR in the pathophysiology of CF. There are over 1,500 mutations in CFTR that fall into one of five categories of mutations. These mutations can cause a breadth of problems ranging from no production of CFTR RNA to abnormal CFTR conductance. The classes of mutations and their phenotypes are summarized in Figure 1.5. In Caucasians, the most abundant genotype is ΔF508 (deletion of phenylalanine at amino acid 508 found in NBD1), occurring in greater than 50% of CF patients, which results in misfolding of CFTR and retention in the endoplasmic reticulum (Class II mutation). While different mutations

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can affect CFTR in a variety of ways, ultimately, the extent to which CFTR is functional correlates with degree of disease severity.

While the majority of research and clinical efforts have focused on pulmonary and gastrointestinal complications, defects in additional systems, including endocrine (67), immunological (46), reproductive (68), muscular (50), and bone (47) have been identified and found to cause significant morbidity associated with CF. Likewise, there are reports of cardiac abnormalities in CF patients, including alterations in left ventricular function, ventricular arrhythmias, tachycardia, cardiomyopathy, myocardial fibrosis and necrosis (69-74).

CONTROVERSY REGARDING CFTR IN THE HEART AND ITS RELATION TO CYSTIC FIBROSIS

The electrophysiological evidence for CFTR being responsible for the PKA-stimulated Cl^- channel in the heart is substantial. Despite these findings, there is significant controversy regarding the functional significance of CFTR in heart function. This controversy is a result of: 1) low CFTR expression in human cardiac tissue (53, 59); 2) conflicting reports about the presence of CFTR currents in human cardiac tissue (58, 59, 75); and 3) rare clinical cardiovascular manifestations in CF patients (personal discussions with CF physicians). It is unclear whether the results with CFTR expression and currents are due to the difficulty in obtaining reliable tissue samples (diseased *vs.* non-diseased, atrial *vs.* ventricular), the reductionist methods employed (single-cell patch clamp), the age studied (pediatric *vs.* adult), or physiological factors. Even though CFTR expression and current in cardiomyocytes appears to be small in contrast to epithelial cells, small changes in conductance in the heart can lead to significant changes in current and function. When examined in more physiological settings,

several studies have found CFTR involved in physiological events (e.g. ischemia) experienced by heart (76, 77). In particular, a recent study examining ABC transporters in ventricular tissue from humans found that CFTR is significantly down-regulated in individuals who have experienced heart failure (78). While documentation of cardiovascular complications in CF patients is sparse, it is present. In living individuals several studies have been conducted to evaluate potential cardiac defects in CF patients. Collectively, these studies have found that cardiac abnormalities can be detected in CF patients, including alterations in left ventricular function, ventricular arrhythmias, tachycardia, and cardiomyopathy (69, 71-74). Additionally, post-mortem analysis of CF patients found myocardial fibrosis and necrosis in a set of CF patients that had died from sudden cardiac arrest (70). Therefore, while CFTR is clearly not an absolutely necessary protein for cardiac function (as CF patients do live), the role of CFTR in the heart remains unknown. As therapies for CF continue to improve and patients begin to lead longer, more active lives, it becomes imperative to determine whether the observed cardiac abnormalities found in CF patients are due to primary cardiac defects or are secondary due to their lung dysfunction. These findings will establish if CF patients are at increased risk for heart disease.

SPECIFIC AIMS

Broadly, the aims of this dissertation are to first determine if CFTR plays a role in cardiac physiology or exists as a redundant protein and then to explore the mechanism(s) in which CFTR may fulfill its role. Additionally, the focus of this work is to understand how cardiac function may be impacted by loss of CFTR activity, as is found in CF. While multiple components are involved in cardiac function we have chosen to investigate its potential role in cardiomyocyte

contraction rate. We feel that the aims outlined below, which will utilize a spectrum of physiological and cell biology techniques, will answer these basic questions and set a foundation for future areas of investigation.

Aim 1: To determine if CFTR plays a role in the regulation of un-stimulated and β-adrenergic stimulated cardiomyocyte contraction rate and understand how acute and chronic loss of CFTR affects cardiomyocyte contraction rate.

Aim 2: To begin to understand the way in which CFTR may functionally interact with other cardiac proteins, we will examine the potential role of MRP4 in cardiomyocyte contraction rate and determine if CFTR is involved in mediating functional responses to cAMP-modulating proteins, such as MRP4 and PDE4.

Aim 3: To further understand the role of CFTR in heart function, we will compare the structure and in vivo function of adult hearts from wildtype and CFTR knockout mice

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FIGURES
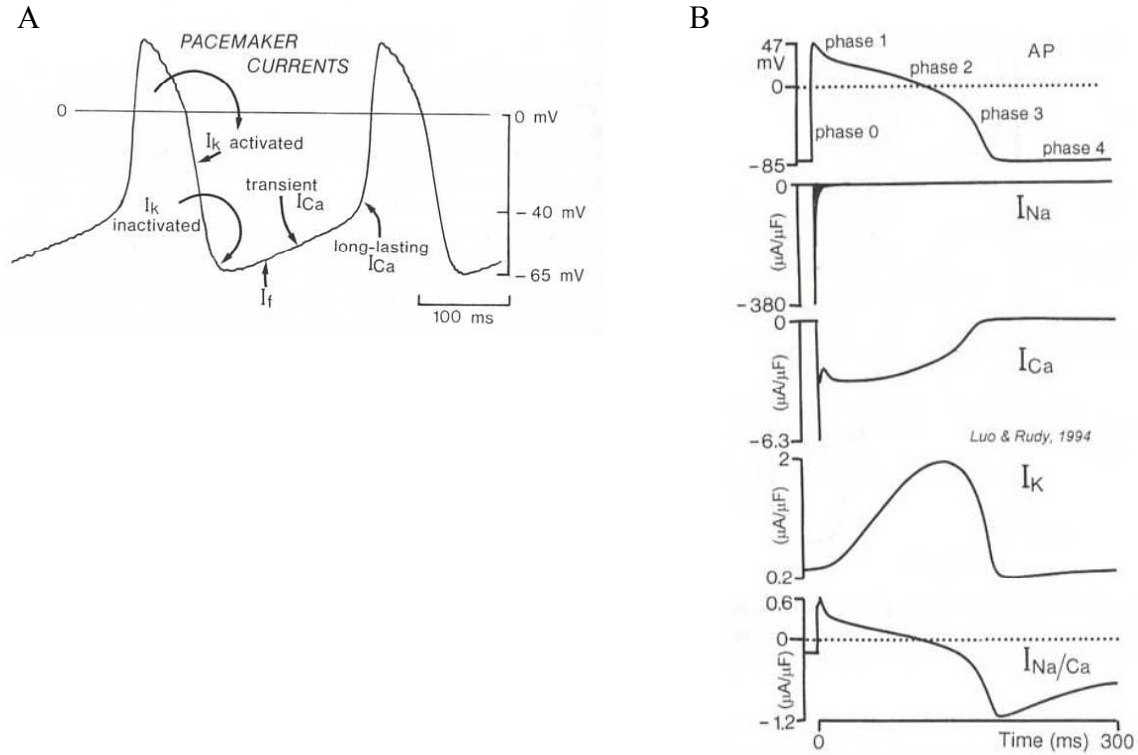
Figure 1.1. Currents responsible for SA nodal (A) and ventricular action potentials (B).
Figures were taken from Opie (3). **A**. "Transient I_{Ca}" refers to T-type Ca^{2+} channels and "long-lasting I_{Ca}" refers to L-type Ca^{2+} channels. **B.** "Na/Ca" refers to the Na^+/Ca^{2+} exchanger.
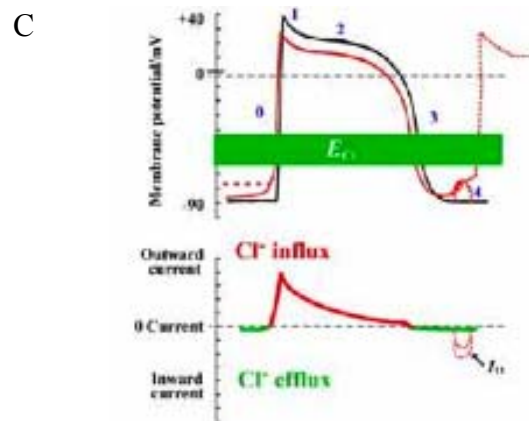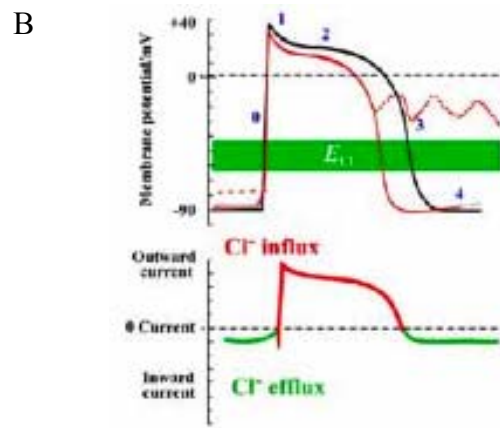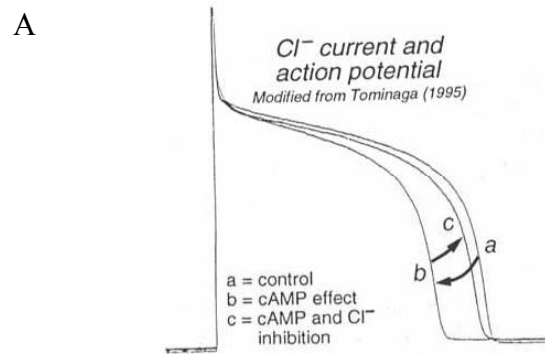
Figure 1.2. Roles of outwardly conducting Cl⁻ currents on β-adrenergic stimulated action potentials. A. General effect of Cl⁻ removal on action potential duration (3). **B and C.** Role of swelling-activated and Ca^{2+}-activate Cl⁻ channels on cardiac action potentials, respectively. Top represents action potential and bottom represents actual current. Black lines indicate normal action potential with red lines indicating effect of current loss (7).
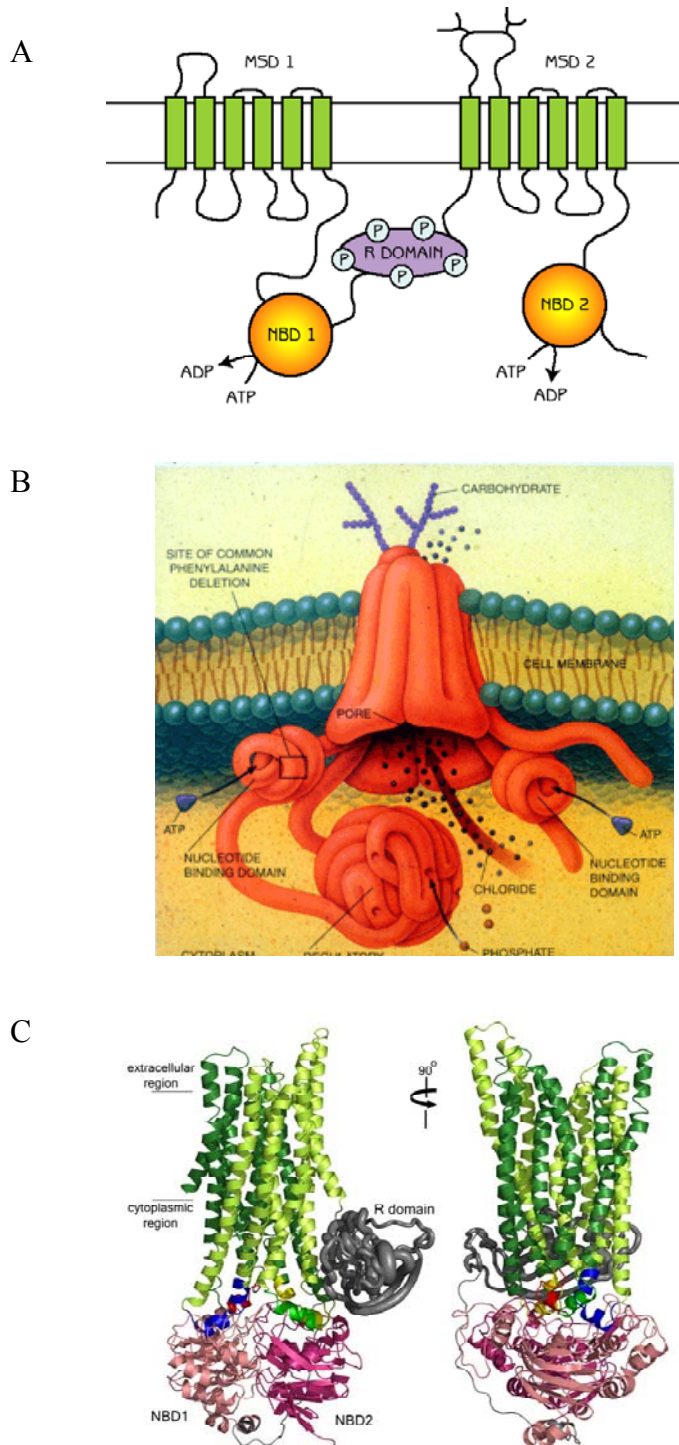
Figure 1.3. Schematics and models of CFTR structure. A. Schematic representation of CFTR, modified from http://physpharm.ohsu.edu/faculty/Dawson%20Lab/Dawson%20Lab%20Homepage.html**B.** Artist rendering of CFTR (79). **C.** Theoretical model of CFTR, modified from Serohijos *et al* (80).

A
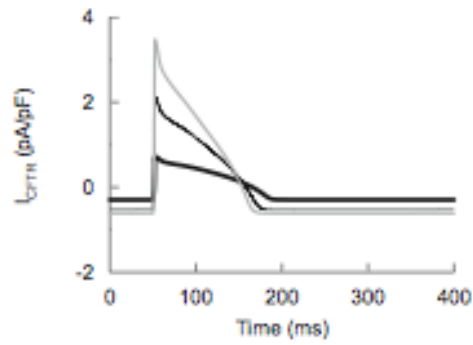
B
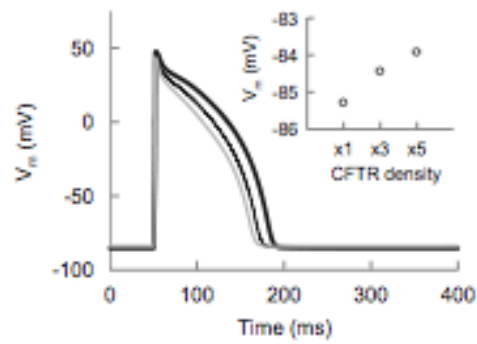
Figure 1.4. Simulation of action potential duration and membrane potential during β-adrenergic stimulation with various densities of CFTR. Thick black, thin black, and gray lines represent the data obtained with 1x, 3x, and 5x CFTR densities, respectively. Figure taken from Kuzomoto *et al* (60).

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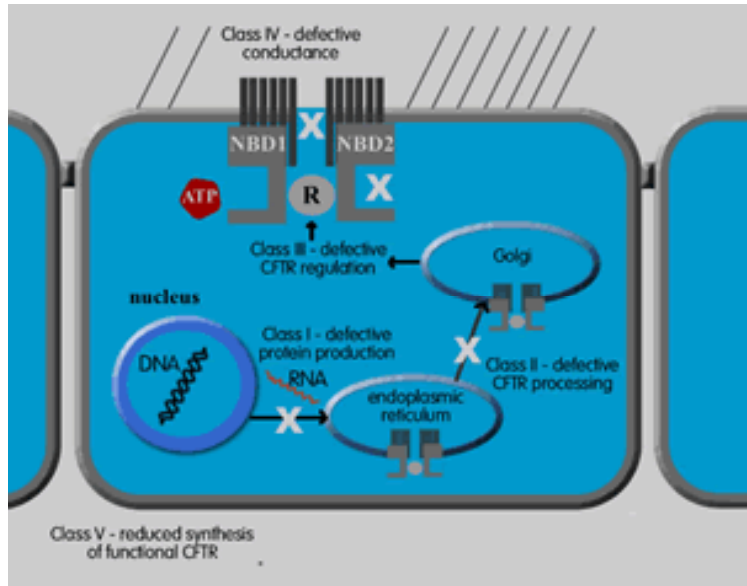
Figure 1.5. Representation of the types of CFTR mutations associated with cystic fibrosis.
Taken from http://www.cysticfibrosismedicine.com/htmldocs/CFText/genetics.htm

CHAPTER 2:

CFTR IN THE REGULATION OF CARDIOMYOCYTE CONTRACTION RATE AND COMPENSATORY ROLES OF CAMKII AND Ca^{2+}-ACTIVATED Cl$^-$ CHANNELS

ABSTRACT

In ventricular cardiomyocytes the cystic fibrosis transmembrane conductance regulator (CFTR) has been extensively studied using electrophysiological techniques, however, there is a paucity of data regarding its physiological function. Using spontaneously beating neonatal ventricular cardiomyocytes from wildtype or CFTR knockout (KO) mice, we examined the role of CFTR in the modulation of cardiomyocyte contraction rate. Contraction rates of spontaneously beating myocytes were captured by video imaging. Real-time changes in Ca^{2+}$_i$ and PKA activity were measured by Fura-2 and fluorescence resonance energy transfer, respectively. Additionally, phosphorylation of phospholamban at threonine 17 (PLB-Thr17) was measured by Western blot. Pharmacological inhibition of CFTR by CFTR$_{inh}$-172 resulted in a transient inhibition of contraction rate, whereas CFTR KO mice displayed normal contraction rates. Further investigation revealed that acute loss of CFTR activity results in a transient increase in Ca^{2+}$_i$ via L-type Ca^{2+} channel activation, resulting in inhibition of PKA activity. Additionally, we found that contraction rate normalization from acute CFTR inhibition or chronic deletion relies on activation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and Ca^{2+}-activated Cl$^-$ channels (CaCC) as simultaneous addition of myristoylated-autocamtide-2-related inhibitory peptide or niflumic acid, with CFTR$_{inh}$-172 or in CFTR KO mice resulted in sustained attenuation of contraction rates. CFTR$_{inh}$-172 also caused significant alterations in PLB-Thr17 phosphorylation. Our results indicate that CFTR is involved cardiomyocyte contraction

regulation, however, loss of CFTR is compensated by activation of CaMKII and CaCC.

Increased dependence on CaMKII upon loss of CFTR may leave cystic fibrosis patients at an

increased risk for heart dysfunction and disease.

INTRODUCTION

To sustain proper physiological function, the heart maintains tight control of ion channels

and transporters. Four major Cl^- currents have been described in the heart, including the cystic

fibrosis conductance regulator (CFTR), Ca^{2+}-activated Cl^- channels (CaCC), swelling-activated

Cl^- channels, and inward rectifying Cl^- channels. As outwardly conducting channels, it has been

thought that CFTR, swelling-activated Cl^- channels, and CaCC share a similar role in

maintaining the resting membrane potential and minimizing action potential prolongation

associated with stimulation of inward Ca^{2+} current. As a result, these Cl^- channels may be

arrythmogenic and prevent hypercontraction through prevention of Ca^{2+}_i overload (1-3).

Shortly after the identification of the CFTR gene from epithelia, a cAMP- or PKA-

dependent Cl^- channel resembling CFTR was identified in cardiomyocytes (4). Subsequently,

expression of a cardiac-specific isoform of CFTR (cCFTR) was reported in atrial and ventricular

myocytes of numerous species, including human (5-8). This isoform arises from alternative

splicing, resulting in loss of a portion of the first intracellular loop, but otherwise retains >95%

sequence homology to epithelial CFTR. cCFTR behaves similarly to epithelial CFTR and is

activated and inhibited by the same agents that affect epithelial CFTR. Additionally, clinical

mutations of CFTR, which cause the disease cystic fibrosis (CF), lie outside of this region.

While no clear physiological role for CFTR in the heart has been firmly established, it has been

suggested that, as a Cl^- channel, CFTR may be involved in the autonomic regulation of action

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potential duration and minimize the action potential prolongation associated with β-adrenergic stimulation, similar to the other outwardly conducting cardiac Cl⁻ channels mentioned previously (9). Studies showing that CFTR is up-regulated during ischemia (10) and that CFTR KO mice lose the protection of ischemic preconditioning (11), suggest the importance of CFTR in maintaining healthy cardiomyocytes during physiological events experienced by the heart. While the aforementioned studies have provided important insight into potential roles of CFTR in the heart, we still lack a full understanding regarding the physiological role(s) of CFTR and the mechanism(s) employed to fulfill its functions.

In an attempt to further understand the role(s) of CFTR in cardiac physiology, we examined the potential role of CFTR in the regulation of ventricular cardiomyocyte contraction rate and the impact of its loss. Our data show that CFTR is involved in the regulation of cardiomyocyte contraction rate, however, acute or chronic loss of CFTR can be compensated for by activation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and Ca^{2+}-activated Cl⁻ channels (CaCC). Acute loss of CFTR causes activation of L-type Ca^{2+} channels, resulting in Ca^{2+}-dependent inhibition of PKA activity and subsequent activation of CaMKII and CaCC, which are required for contraction rate recovery. Similarly, we found that CFTR KO mice retain normal contraction rates through an increased dependence on CaMKII and CaCC activities. Illuminating the role of CFTR in cardiomyocyte physiology will enhance our knowledge regarding normal cardiac processes, while understanding the effects of loss of CFTR activity on cardiac processes will help to determine if CF patients are at increased risk for cardiac defects.

MATERIALS AND METHODS

Ethical approval of animal use. FV/B mice were obtained from Charles River

Laboratory (Wilmington, MA) and used for generation of wildtype (WT) mice. CFTR KO mice

(strain 2515 B6.129S6-*Cftr*^*tm1Kth*/J) were initially obtained from Dr. Deborah Nelson at the

University of Chicago (Chicago, IL) and bred in-house. All animals were used within 24 hours

of birth. In the current study a total of 191 WT and 54 CFTR KO mice were used. Use of

animals was approved by the University of Illinois at Urbana-Champaign IACUC.

Isolation and culture of neonatal mouse cardiomyocytes. Ventricular myocytes were

cultured from neonatal mice for all experiments using a similar protocol as previously reported

(12). Freshly born mice were sacrificed by decapitation with surgical scissors. Decapitation was

used so that isolated myocytes would not be affected by anesthetics or altered environment

(redox and pH (CO_2)). This procedure is justified to yield viable myocytes after isolation as both

sedation and anesthetics significantly reduce the survival rate of isolated heart cells from

neonates. Decapitation of neonates avoiding prior anesthesia is done in compliance with the

Report of the AVMA Panel on Euthanasia JACMA, Col 218, No. 5, March 1, 2001 p 682-3

(http://www.avma.org/resources/euthanasia.pdf), and the NIH ARAC Guidelines for the

Euthanasia of Mouse and Rat Fetuses and Neonates

(http://oacu.od.nih.gov/ARAC/euthmous.htm). Neonatal mouse cardiac tissue devoid of atria

were exposed to enzymatic digestion. Subsequently, isolated cells were pipetted onto 35 mm

Petri dishes pre-coated with 1.5% gelatin type A or laminin. Cells were plated at high- and low-

density for measurement of contraction rate or real-time imaging experiments, respectively.

High-density plating allowed for cells to form a syncytium, whereas low-density plating

facilitated individual quiescent myocytes. Quiescent myocytes were used for imaging

experiments to eliminate any confounding effects from signaling associated with the action of contraction. Cells were incubated at 37°C for 2-3 days until they displayed appropriate myocyte morphology or formed a syncytium for contraction rate measurements. Culture media (containing serum and 20 mM HEPES for buffering) was replaced daily and at least 1 hour prior to experiments.

Measurement of contraction rate. Measurement of myoctye contraction rate was performed as previously reported (13). 35 mm dishes were placed in a temperature/CO_2 regulated apparatus. Using a CCD camera and MetaMorph imaging software, 5-second video images were obtained every 2 minutes. Following acquisition, a specific point within the field was selected and analyzed for movement in each x, y, and z plane. Movements in the x and y plane were calculated and number of deflections noted as number of contractions. Each data point represents a single observation from different experiments so that only one data set was obtained from each experiment. Each drug was tested using multiple isolations to ensure that the observed responses were not particular to a single isolation.

Measurement of Ca^{2+}_i. Quiescent ventricular myocytes were cultured at low-density as described above. Upon formation of myocyte morphology (24-48 hours after culture), culture media was removed and cells were washed with 1X dulbecco's phosphate buffer solution (DPBS). After which cells were loaded with 5 μM Fura-2 AM dissolved in 0.01% Pluronic F-127 plus 0.1% DMSO in DPBS at room temperature for 30 minutes. Following loading, cells were washed with 1X DPBS or Ca^{2+}-free DPBS (with EGTA (500 μM) to remove residual free Ca^{2+}) and allowed to sit for 30 minutes to remove any unloaded dye and allow for complete de-esterification of Fura-2 AM into Fura-2, its active form. For Ca^{2+}-free experiments, experiments were performed in Ca^{2+}-free DPBS with EGTA. Changes in Ca^{2+}_i were monitored by Fura-2

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excitation at 340nm and 380nm and emission at 500nm. As the ratio of the light emitted at 340nm excitation to that emitted at 380nm excitation (340/380 ratio) is a direct index of the level of Ca^{2+}_i, data is expressed as the 340/380 ratio following subtraction of background fluorescence.

Measurement of real-time PKA or cAMP activity via fluorescent resonance energy transfer (FRET). Following 24 hours in culture, neonatal cardiomyocytes were infected with an adenovirus containing AKAR2.2 (PKA) or ICUE3 (cAMP) (100 MOI, 24 hours) in a similar manner as previously published (14). 24-48 hours post-infection cells were washed and stored in DPBS for FRET recordings. Dual emission ratio imaging was acquired for 200 seconds at 20-second intervals. Images in both channels were subjected to background subtraction and ratios of yellow-to-cyan were calculated at different time points and then normalized to baseline signals. For AKAR2.2, phosphorylation of the PKA consensus site leads to an increase in the YFP/CFP ratio (14), whereas the binding of cAMP to the EPAC site of ICUE3 causes a decrease in FRET (15).

Measurement of phospholamban phosphorylation. Neonatal cardiomyocytes, pretreated with 20 μM CFTR$_{inh}$-172 for various times, were chilled, washed, and harvested in lysis buffer (20 mM Hepes pH 7.5, 2 mM EDTA pH 8.0, 150 mM NaCl, 10% Glycerol, 0.6% NP_4O, 20 mM $Na_4P_2O_7$, 50 mM NaF, 2.5 mM Na_3VO_4 and Protease Inhibitor Single-Use Cocktail (Thermo Scientific, PIERCE)). The lysates were clarified at 13,200 x *g* for 20 minutes at 4° C. The samples were resolved on a SDS-PAGE gel. Phospholamban phosphorylation was detected with a phospho-Threonine 17 antibody (PLB-Thr17, Badrilla, UK). Additionally, total phospholamban was probed with an anti-phospholamban antibody (PLB, Badrilla, UK) and γ-tubulin with an anti-γ-tubulin antibody (Sigma, MO). Primary antibodies were visualized with IRDye 680CW goat anti-mouse or with IRDye 800CW goat anti-rabbit secondary antibodies

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using an Odyssey scanner (Li-cor biosciences, NE). The signals obtained from PLB-Thr17 were corrected and plotted as the increase over internal control (γ-tubulin).

Chemicals. Niflumic acid, thapsigargin, and myristoylated-PKA inhibitor fragment 14-22 (PKI) were obtained from Sigma-Aldrich (St. Louis, MO). CFTR$_{inh}$-172 and BayK-8644 were obtained from Calbiochem (San Diego, CA) and Cystic Fibrosis Foundation Therapeutics (CFTR$_{inh}$-172 only)(Chicago, IL). Myristoylated-autocamtide-2-related inhibitory peptide (AIP) was obtained from Biomol (Plymouth Meeting, PA). Fura-2 AM was purchased from Invitrogen (Carlsbad, CA). Ryanodine came from Alomone Labs (Jerusalem, Israel). Niflumic acid, CFTR$_{inh}$-172, ryanodine, thapsigargin, and Fura-2 AM were dissolved in DMSO, while PKI and AIP were dissolved in water. All drugs were made as 1:1000 stock solutions and diluted the day of the experiment.

Statistical analysis. For presentation of changes in contraction rate, Ca^{2+}_i, or PKA activity, peak responses were subtracted from their respective baseline values. For percentage baseline measurements, the last measured contraction rates were divided by baseline values. Normalization was performed by GraphPad Prism software by setting the first baseline measurement to 100% and the value of 0 to 0%. For comparison between two groups of data, student's t-test was used to determine significance. For multiple comparisons, one-way analysis of variance (ANOVA) was performed. Comparison of time course curves were performed by two-way ANOVA. Results were considered significant at $P < 0.05$ and are denoted accordingly.

RESULTS

Effect of pharmacological inhibition and genetic deletion of CFTR on cardiomyocyte contraction rate. To examine the potential role of CFTR in the regulation of

ventricular cardiomyocyte contraction rate, we used syncytial neonatal ventricular myocytes, which form a single spontaneously beating unit. In control experiments (DMSO or H_2O) we found that cardiomyocytes held a steady rhythm of beating over time (Figures 2.1, 2.4, 2.5). To determine the effect of CFTR inhibition on cardiomyocyte contraction rate, we used the selective CFTR inhibitor, $CFTR_{inh}$-172. Application of $CFTR_{inh}$-172 (10 and 20 μM) resulted in a transient, but significant ($P < 0.05$), dose-dependent inhibitory effect of contraction rate that subsequently returned to baseline levels (Figure 2.1). Addition of $CFTR_{inh}$-172 (20 μM) to CFTR KO myocytes produced no significant inhibition of contraction rate compared to baseline or DMSO ($P > 0.05$), indicating specificity of action of $CFTR_{inh}$-172 and the ability of CFTR KO cardiomyocytes to function normally without CFTR. Thus, we found that CFTR is involved in the physiological regulation of cardiomyocyte contraction rate; however, both acute and chronic loss of CFTR activity can be compensated for.

Effect of $CFTR_{inh}$-172 of Ca^{2+}_i and real-time PKA activity. Previously, the contraction rate of spontaneously beating cardiomyocytes has been shown to be dependent on changes in Ca^{2+}_i and PKA activity (16). Therefore, in an effort to understand the mechanism whereby CFTR inhibition causes a transient inhibition in cardiomyocyte contraction rate we investigated if $CFTR_{inh}$-172 alters Ca^{2+}_i and/or PKA activity. We first examined Ca^{2+}_i by monitoring changes in Fura-2 fluorescence, which is a direct index of Ca^{2+}_i, in WT and CFTR KO mice. In WT mice, $CFTR_{inh}$-172 (20 μM) caused a significant increase in Ca^{2+}_i compared to DMSO treated myocytes ($P < 0.001$) (Figure 2.2A). In contrast, in CFTR KO mice $CFTR_{inh}$-172 failed to elicit a significant increase in Ca^{2+}_i compared to DMSO ($P > 0.05$). To understand the source of this Ca^{2+}_i increase we performed additional experiments targeting extracellular and/or intracellular Ca^{2+} stores and the channels/transporters responsible for our observed effects. As

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seen in Figure 2.2B, removal of extracellular Ca^{2+} completely prevented the CFTR$_{inh}$-172 induced increase in Ca^{2+}_i. In contrast, inhibition of sarcoplasmic reticulum Ca^{2+} release by pretreatment with thapsigargin (1 μM) & ryanodine (10 μM) prior to CFTR$_{inh}$-172 did not have any significant influence on Ca^{2+}_i changes from CFTR$_{inh}$-172. While many routes of Ca^{2+} entry in cardiomyocytes exist, L-type Ca^{2+} channels and the Na^+/Ca^{2+} exchanger are the most prominent. Therefore, we examined if one or both of these proteins are involved in mediating CFTR$_{inh}$-172 induced increases in Ca^{2+}_i. Pretreatment with the L-type Ca^{2+} channel inhibitor nifedipine (10 μM), but not the Na^+/Ca^{2+} exchange inhibitor KB-R7943 (10 μM) completely prevented CFTR$_{inh}$-172-induced increases in Ca^{2+}_i. Thus, these data indicate that inhibition of CFTR causes L-type Ca^{2+} channel activation resulting in a transient increase in Ca^{2+}_i.

Subsequently, we sought to determine the effect of CFTR$_{inh}$-172 on PKA activity. To do this, we utilized FRET to monitor real-time PKA activity. Compared to DMSO, CFTR$_{inh}$-172 (20 μM) resulted in a significant inhibition of PKA activity, both in magnitude and time ($P < 0.001$)(Figure 2.3A). This inhibition was transient and recovered to baseline levels within 20 minutes, resembling the pattern of inhibition observed on contraction rates. This effect was not the result of upstream alterations in adenylyl cyclase activity as separate experiments measuring cAMP showed that CFTR$_{inh}$-172 caused no change in cAMP levels (0.17 ± 0.02 *vs.* 0.20 ± 0.01 - ΔYFP/CFP, $P > 0.05$ *vs.* DMSO). To understand how CFTR inhibition leads to a decrease in PKA activity we examined if there was a link between the previously observed Ca^{2+}_i change and PKA activity. In these experiments, inhibition of the L-type Ca^{2+} channel with nifedipine pretreatment (10 μM) completely prevented CFTR$_{inh}$-172-induced attenuation of PKA activity (Figure 2.3B), indicating that activation of L-type Ca^{2+} channels was responsible for CFTR$_{inh}$-172-induced inhibition of PKA activity. Similarly, direct activation of L-type Ca^{2+} channels by

BayK-8644 (1 µM) caused a significant decrease in PKA activity, providing further evidence

that L-type Ca^{2+} activation can negatively affect PKA activity. To verify that inhibition of PKA

activity would directly lead to functional attenuation of contraction rate, we examined the effect

of PKI (20 µM), a selective peptide inhibitor of PKA, on cardiomyocyte contraction rate. In

agreement with previous reports (14, 16), we found that PKA inhibition caused a significant

decrease in contraction rate (P < 0.001)(Figure 2.3C). In separate experiments, we found that

direct activation of L-type Ca^{2+} channels by BayK-8644 (1 µM) also led to a significant

inhibition of contraction rate (-133 ± 5 Δbeats/min, P < 0.001 *vs.* H_2O, n = 6). Taken together

with our previous findings, these results indicate that inhibition of CFTR causes a transient

increase in Ca^{2+}_i via activation of L-type Ca^{2+} channels and that this causes a transient inhibition

of PKA activity, thereby leading to a decrease in contraction rate.

CaMKII in the recovery of CFTR$_{inh}$-172-induced contraction rate depression.

Previously it has been shown that PKA and CaMKII regulate overlapping signaling cascades

leading to complimentary regulation of proteins (i.e. phospholamban) involved in cardiomyocyte

contraction (17). With our observed increase in Ca^{2+}_i, we sought to determine if CFTR

inhibition altered CaMKII activity. We first examined the effect of CaMKII inhibition on

cardiomyocyte contraction rate. In WT mice, addition of AIP (1 µM), a selective CaMKII

inhibitor, alone reproducibly caused a minor, statistically insignificant (P > 0.05) decrease in

contraction rate compared to control (Figure 2.4A). However, in separate experiments, when we

added CFTR$_{inh-172}$ (20 µM) together with AIP, cardiomyocyte contraction rate was not able to

recover to baseline in a manner comparable to CFTR$_{inh}$-172 alone (Figure 2.4B). To further

examine this we measured the effect of CFTR$_{inh}$-172 on PLB-Thr17 phosphorylation, a specific

CaMKII phosphorylation site on phospholamban. In agreement with our functional data,

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CFTR$_{inh}$-172 (20μM) caused a significant increase in PLB-Thr17 phosphorylation (Figure 2.4C). This phosphorylation occurred very rapidly (within 2 minutes) and was transient as phosphorylation returned to near-baseline levels within 4 minutes. Thus, while CFTR$_{inh}$-172 induces a transient increase in Ca^{2+}$_i$ that causes a decrease in contraction rate, it also causes activation of CaMKII, which facilitates recovery from CFTR inhibition. To determine if CaMKII is also important under conditions of chronic CFTR loss, we examined the effect of CaMKII inhibition in CFTR KO mice. As seen in Figure 2.4D, inhibition of CaMKII by AIP produced a profound inhibitory effect on CFTR KO contraction rate. This effect was significantly enhanced from the effect of AIP on WT cardiomyocytes (Figure 2.4E). Therefore, whereas CFTR KO cardiomyocytes produce normal contraction rates, they appear to have an increased dependence on CaMKII activity to maintain these rates.

Role of CaCC in the regulation of cardiomyocyte contraction rate. Subsequently we sought to determine how CaMKII activation could facilitate an increase in contraction rate for recovery from CFTR inhibition. Due to the transient nature of PLB-Thr17 phosphorylation we propose that this recovery was not purely a result of altered PLB phosphorylation. It has been widely shown that loss of CFTR activity can lead to increased activation of CaCC as a compensatory Cl$^-$ conductive pathway(18). With our observations that CFTR inhibition increases Ca^{2+}$_i$ and CaMKII activity, we sought to determine if activation of CaCC could compensate for loss of CFTR activity. To investigate CaCC in cardiomyocyte contraction rate regulation, we used niflumic acid (100 μM) to directly inhibit CaCC. Addition of niflumic acid alone resulted in a rapid, but transient, decrease in contraction rate, which recovered to baseline (Figure 2.5A). Thus inhibition of either CFTR or CaCC resulted in similar functional effects on cardiomyocyte contraction rate indicating that, in ventricular cardiomyocytes, CFTR and CaCC may have

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complimentary roles. To determine if CaCC activity is necessary for recovery from CFTR inhibition, we performed experiments in which we simultaneously applied niflumic acid (100 µM) and CFTR$_{inh}$-172 (20 µM). We found that inhibition of both CFTR and CaCC resulted in an initial abolishment of contraction rate, which was no longer able to fully recover to baseline (Figure 2.5B). Subsequent examination of CaCC inhibition in CFTR KO mice also showed a significant inhibitory effect of niflumic acid on cardiomyocyte contraction rate. Similar to AIP, the inhibitory effect of niflumic acid in CFTR KO mice was significantly enhanced to that in WT mice (Figure 2.5C and 2.5D), indicating an increased reliance on CaCC. These results indicate that CaCC are important in recovery from CFTR inhibition and constitute an important downstream effector of CaMKII activation.

DISCUSSION

Since the first electrophysiological recording of CFTR in the heart in 1992 (19), there has been little dispute regarding it being responsible for the PKA-stimulated Cl$^-$ channel in the heart. While there have been conflicting reports about CFTR expression and/or activity in humans (20-22), it has been found that CFTR is expressed and electrophysiological recordings made from rats (23), guinea pigs (24), rabbits (25), swine (26), dogs (24), primates (21), and most pertinent to our study, mice (27). Despite these studies, questions remain regarding the role of CFTR in the heart. Thus, the primary goal of our study was to determine if CFTR plays a physiological role in the regulation of heart function. Using a selective pharmacological inhibitor of CFTR we have shown that CFTR does play an active role in the regulation of heart function, as measured by CFTR$_{inh}$-172 induced depression of cardiomyocyte contraction rate. These results are in agreement with cardiomyocyte simulation studies which showed that decreased CFTR current

density would lengthen action potential duration (28). In our study we found that inhibition of CFTR causes a transient increase in $Ca^{2+}{}_i$ due to activation of L-type Ca^{2+} channels, whose importance in action potential duration and contraction regulation are well established. Future dissection of the nature of the interactions (i.e. direct or indirect) between CFTR and L-type Ca^{2+} channels may provide important insights into the regulation of cardiomyocyte contraction.

In addition to alterations in $Ca^{2+}{}_i$, we have also shown that CFTR inhibition leads to a transient decrease in PKA activity, as measured by A-kinase activity receptor (AKAR) phosphorylation. This study, along with others' (14, 16), have shown that PKA is an important regulator of contraction. We did not attempt to dissect out the exact mechanism whereby CFTR inhibition leads to PKA inhibition, however, we did show that this inhibition was caused by L-type Ca^{2+} channel influx. Cardiac myocytes contain Ca^{2+}-sensitive adenylyl cyclases (AC5, AC6) that can be inhibited by increases in $Ca^{2+}{}_i$ (29). However, our findings that cAMP remains unchanged following CFTR inhibition rules out this possibility. One possibility is that Ca^{2+}-sensitive phosphatases, such as calcineurin, are involved in the inhibition we observed. It has been shown that calcineurin antagonizes PKA activity through dephosphorylation of PKA effectors (30) and even direct inhibition of PKA itself (31). Additionally, with the lack of effect on cAMP, we would argue that CFTR inhibition likely does not alter hyperpolarization cyclic-gated nucleotide channels (HCN, I_f), which are known to be involved in contraction rate regulation (unpublished results)(32); although we cannot rule out an indirect effect of membrane potential alterations. Altogether, we have documented how an inhibition of CFTR activity can alter ion channel and intracellular signaling activities to affect cardiomyocyte function.

However, importantly, we found that CFTR-dependent depression of contraction rate was transient and was restored to normal rates. Additionally, we found that CFTR KO mice

displayed normal contraction rates, further indicating that CFTR activity or expression is not critical to maintaining cardiomyocyte function. The fact that CFTR is not critical to maintaining baseline contraction rate is not surprising given that mutations in CFTR do not lead to early embryonic death in CF patients or mice. The ability of cardiomyocytes to compensate for transient or long-term loss of CFTR does not discount its significance, but rather underscores the adaptability of the heart to maintain its vital function. It is well accepted that gene knockout causes adaptation, but we have shown that short-term and chronic inhibition of CFTR leads to compensation in cardiac myocytes. In our current study we have found that CaMKII and Ca^{2+}-activated Cl^- channel (CaCC) activity was necessary for recovery from acute CFTR inhibition and maintenance of normal contraction rates in CFTR KO mice. An interplay between CFTR and CaCC has been noted in several tissue types that express both channels. In the airway, loss of CFTR, either in CF patients or CFTR KO mice, leads to increased activity of CaCC to compensate for loss of CFTR-dependent Cl^- current (33). Additionally, in bovine pulmonary artery endothelial cells, *Xenopus* oocytes, and mouse parotid acinar cells, expression of CFTR leads to a negative regulation of CaCC (33). While the CaCC inhibitor we used, niflumic acid, is a well-documented inhibitor of CaCC (33), it is not specific as previous studies have shown that niflumic acid can also affect CFTR (34) and K^+ channels (35, 36). While we cannot rule out effects on non-CFTR targets, our data showing that niflumic acid causes significant inhibition of contraction rate in CFTR KO mice makes us confident that our results are not due to additional effects on CFTR. This coupled with the involvement of Ca^{2+}_i and CaMKII, both ways in which CaCC are activated, strengthens our view about the importance of CaCC during loss of CFTR activity. However, since Ca^{2+} and CaMKII can also affect many other ion channels and kinases

important in contraction, it is necessary to further explore how CFTR inhibition in cardiac myocytes may affect other cellular components.

CaMKII has been identified as an important intracellular signaling molecule in cardiac myocytes involved in regulating numerous cellular processes, including activation of ion channels, modulation of action potential, hypertrophy, and apoptosis (37). In our study we found that CaMKII is important in the recovery of cardiomyocyte contraction rate caused by inhibition of CFTR. Its role in this process appears to be, in part, due to its ability to activate CaCC, as inhibition of CaCC or CaMKII prevented recovery from CFTR inhibition. CaMKII has also been shown to decrease the inactivation rate of L-type Ca^{2+} channels (38), which may aid in contraction rate recovery. Another important finding is that, while CFTR KO mice display normal baseline contraction rates, in the presence of CaMKII inhibition, these rates were severely diminished. In contrast, CaMKII inhibition in WT mice showed only minimal inhibition of baseline contraction rate. These data indicate that CFTR KO mice have an increased dependence on CaMKII. These findings are of particular interest, as cardiac myocytes with increased expression or activity of CaMKII are prone to pathological cardiac conditions. CaMKII has been linked to heart failure; hearts from mice with heart failure have increased expression of CaMKIIδ and overexpression of CaMKII causes heart failure in mice (39, 40). In addition to heart failure, increased CaMKII activity may lead to prolonged action potential duration and cause long QT syndrome by increasing late-inactivating or slowly inactivating Na^+ currents (41). At this time we cannot say whether the increased dependence on CaMKII is due to long-term loss of CFTR activity or loss of CFTR protein, however, it would be intriguing to determine whether CFTR mutations, such as ΔF508 or G551D, which cause decreased protein expression due to abnormal folding or decreased CFTR gating, respectively, also display

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increased dependence on CaMKII activity. Thus, our findings coupled with those by Solbach *et al* that CFTR is downregulated in ventricular tissue from heart failure patients (42), and several observations that heart damage occurs in some CF patients (43, 44) warrants future investigation of the role of CaMKII in CF hearts.

As the most common lethal genetic disorder among Caucasians, CF affects nearly 30,000 individuals per year in the U.S. (www.cff.org). Since the discovery that the primary defect in CF involves mutations in the gene encoding for CFTR, extensive research efforts have been dedicated to characterizing the role of CFTR in the pathophysiology of CF. In addition to the classic systems affected in CF (pulmonary, gastrointestinal, reproductive) recent evidence has shown that CFTR defects lead to pathologies in other organs, including muscle and bone. Likewise, there have been scattered reports of cardiac abnormalities in CF patients, including alterations in left ventricular function, tachycardia, cardiomyopathy, myocardial fibrosis and necrosis (45-48). More recently it was found that 51% of CF patients with severe lung infection had significantly decreased ejection fraction during exercise (49). It is unclear whether these are due to primary cardiac defects or secondary effects from altered pulmonary function. The use of CFTR KO mice may be advantageous in this pursuit as they display a normal pulmonary phenotype, thus allowing dissection of primary *vs.* secondary effects of loss of CFTR. The physiological relevance of CFTR in the heart requires investigation as recent advances in therapies for CF have extended the average lifespan of CF patients to the mid-30's, with some individuals with milder mutations achieving normal life spans (70-80 years). Therefore, it is likely that as therapeutic advances continue to be made and individuals live longer, more active lives, CF patients will enter the age range where cardiovascular abnormalities become apparent and may exhibit significant clinical cardiac symptoms. A more complete understanding of the

role of CFTR in the heart may lead to the identification of potential cardiac defects in CF

patients and thus increase the quality of care for these patients.

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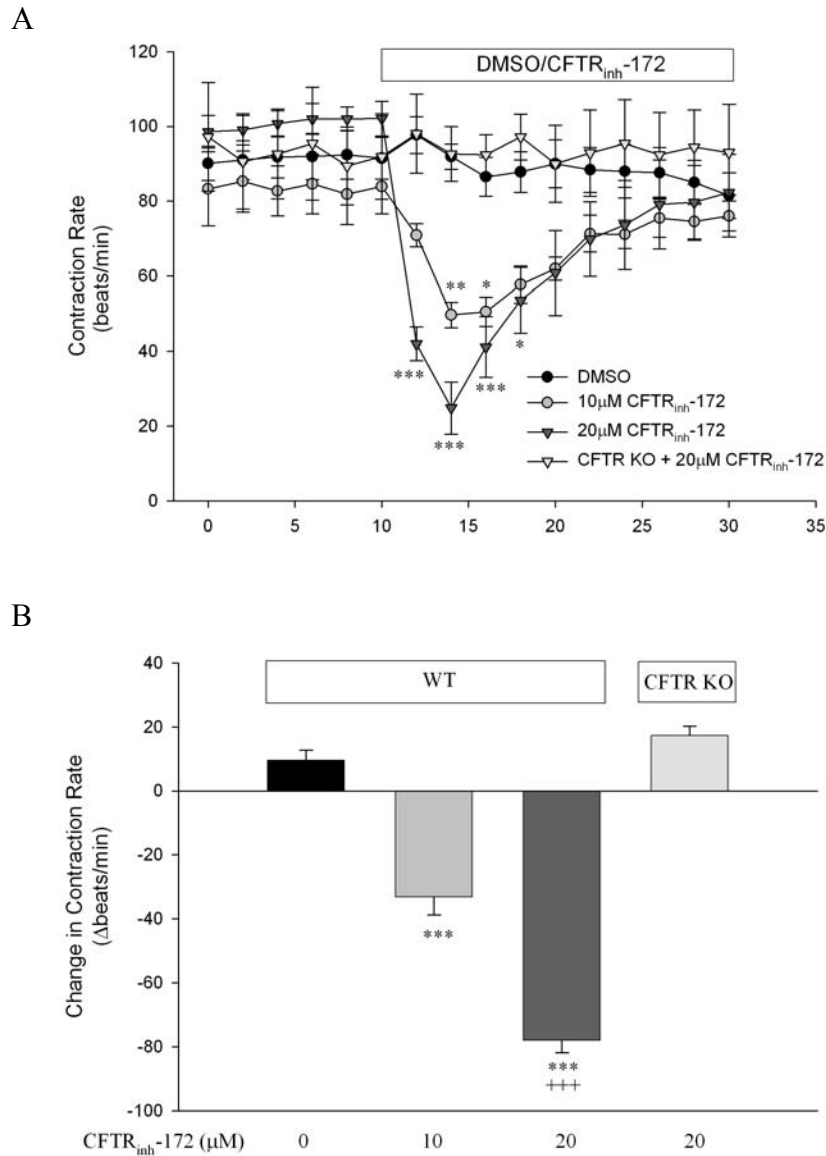
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A

B

Figure 2.1. Pharmacological inhibition, but not genetic deletion, of CFTR attenuates cardiomyocyte contraction rate. A. Syncytial WT ventricular myocytes were treated with DMSO ($n \geq 6$), or CFTR$_{inh}$-172 (10 μM or 20 μM, n = 7 each) for 20 minutes following a 10-minute baseline. Separately, the effect of CFTR$_{inh}$-172 (20 μM, n = 5) on ventricular myocytes obtained from CFTR KO mice was also examined. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* DMSO or CFTR KO by two-way ANOVA. **B.** Comparison of the maximal inhibitory effect of CFTR$_{inh}$-172 on WT and CFTR KO cardiomyocyte contraction rate. ***, $P < 0.001$ *vs.* DMSO by one-way ANOVA; +++, $P < 0.001$ *vs.* 10 μM CFTR$_{inh}$-172 by student's t-test.
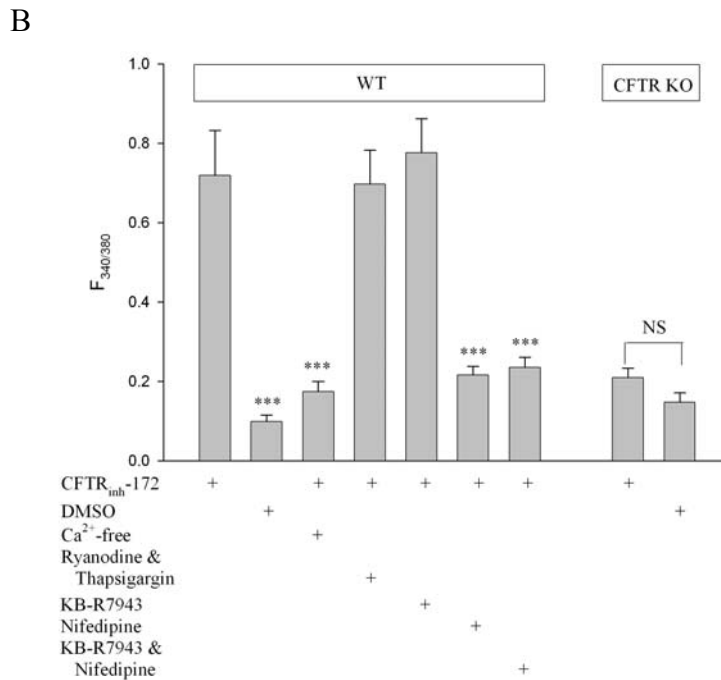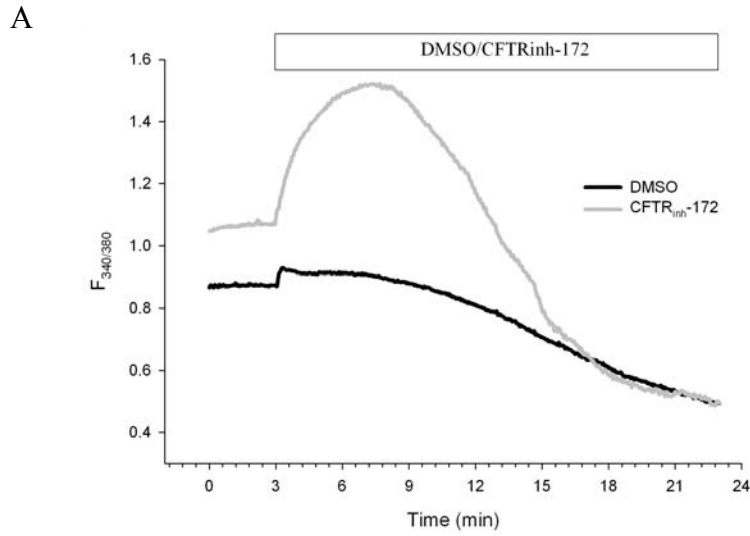
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Figure 2.2. Inhibition of CFTR increases Ca^{2+}_i via L-type Ca^{2+} channels. A. Quiescent WT ventricular myocytes were loaded with Fura-2 to monitor changes in Ca^{2+}_i. Following a 3-minute baseline, $CFTR_{inh}$-172 (20 μM, n = 32) or DMSO (n = 19) was added and subsequent effects were measured for 20 minutes. **B.** WT cardiomyocytes were bathed in Ca^{2+}-free solution (n = 25), pretreated with ryanodine (10 μM) and thapsigargin (1 μM) (n = 26), KB-R7943 (10 μM, n = 23), nifedipine (10 μM, n = 26), or KB-R7943 and nifedipine (n = 23) for 30 minutes prior to Ca^{2+}_i measurements. Additionally, in CFTR KO myocytes, Fura-2 fluorescence was monitored upon $CFTR_{inh}$-172 (20 μM, n = 23) or DMSO (n = 24) addition. *******, P < 0.001 *vs.* $CFTR_{inh}$-172 by student's t-test.
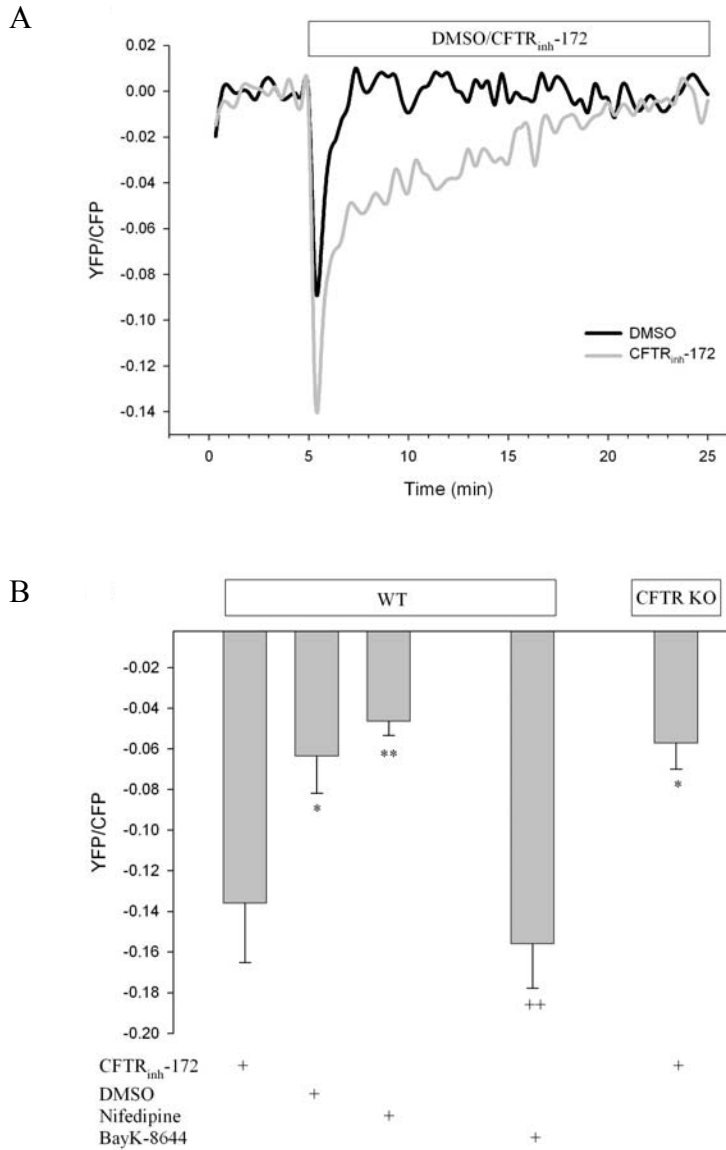
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Figure 2.3. CFTR$_{inh}$-172-induced activation of L-type Ca^{2+} channels causes inhibition of PKA and contraction rate. A. Quiescent WT ventricular myocytes were infected with AKAR2.2 to monitor real-time changes in PKA activity. Following a 5-minute baseline, CFTR$_{inh}$-172 (20 μM, n = 24) or DMSO (n = 18) was added and subsequent effects were measured for 20 minutes. **B.** WT cardiomyocytes were pretreated with nifedipine (10 μM, n = 24) for 30 minutes prior to CFTR$_{inh}$-172 addition. Additionally, PKA activity following CFTR$_{inh}$-172 (20 μM, n = 19) was monitored in CFTR KO myocytes. In separate experiments, the effect of BayK-8644 (1 μM, n = 15) on PKA activity in WT cardiomyocytes was measured. **C.** Syncytial WT ventricular myocytes were treated with PKI (20 μM, n = 8) for 30 minutes following a 10-minute baseline. *, P < 0.05; **, P < 0.01 *vs.* CFTR$_{inh}$-172; ++, P < 0.01 *vs.* DMSO by student's t-test.

Figure 2.3 continued…

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B
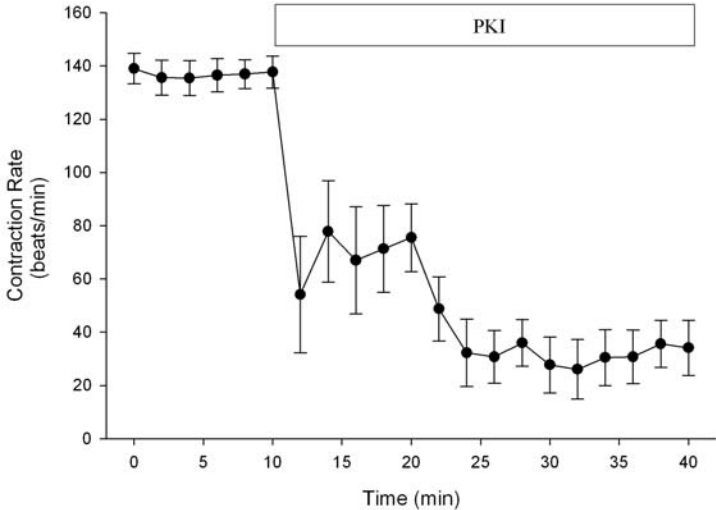
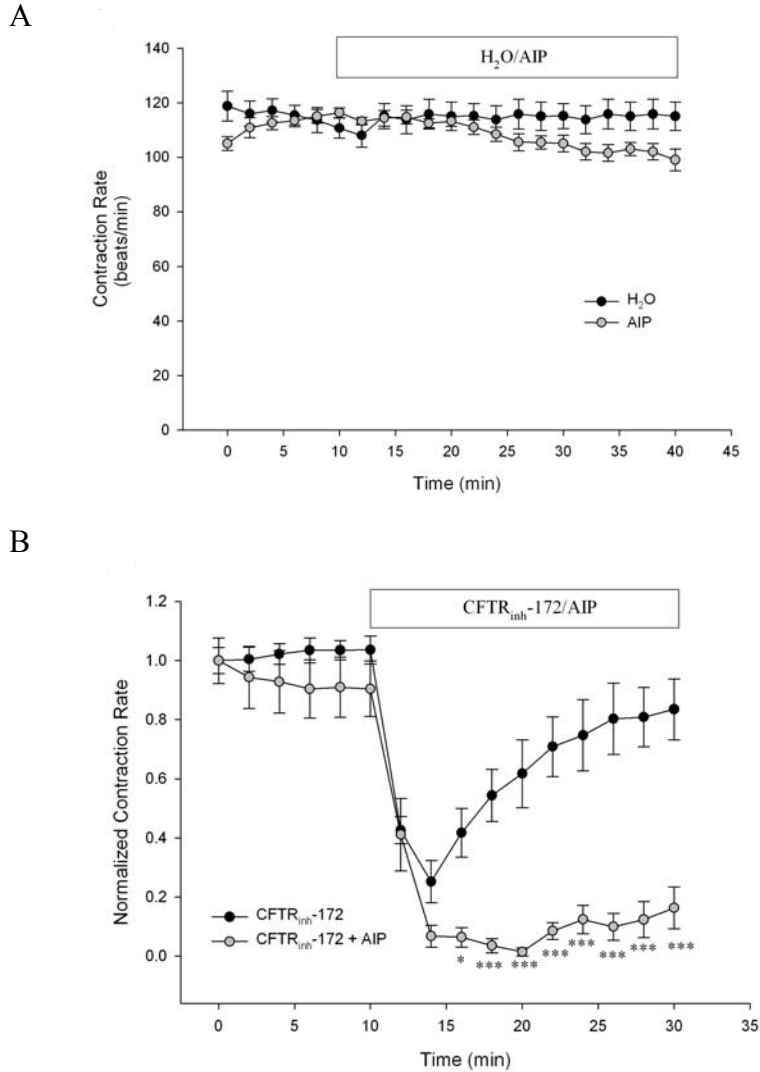**Figure 2.4. CaMKII activity is required to maintain normal contraction rates following pharmacological inhibition or genetic deletion of CFTR. A.** Syncytial WT ventricular myocytes were treated with H_2O (n = 5) or AIP (1 μM, n = 6) for 30 minutes following a 10-minute baseline. **B.** Separately, the effect of simultaneous addition of AIP plus $CFTR_{inh}$-172 (20 μM, n = 8) for 20 minutes was examined and compared to responses by $CFTR_{inh}$-172 alone (from Figure 1A). *, $P < 0.05$; ***, $P < 0.001$ *vs.* $CFTR_{inh}$-172 alone by two-way ANOVA. **C.** WT cardiomyocytes were exposed to $CFTR_{inh}$-172 for various lengths of time (n = 3-5 each) and then harvested to examine PLB-Thr17 phosphorylation. All results were quantified and normalized. A representative blot is shown. *, $P < 0.05$ *vs.* no treatment by student's t-test. **D.** Cardiomyocytes from CFTR KO mice (n = 8) were treated with AIP (1 μM) for 30 minutes following baseline measurements. Contraction rates were compared to those from CFTR KO mice in Figure 1A. *, $P < 0.05$; **, $P < 0.01$ *vs.* CFTR KO alone by two-way ANOVA. **E.** A comparison of changes in contraction rate due to AIP in WT and CFTR KO mice. **, $P < 0.01$ *vs.* WT by student's t-test.

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Figure 2.4 continued…

C
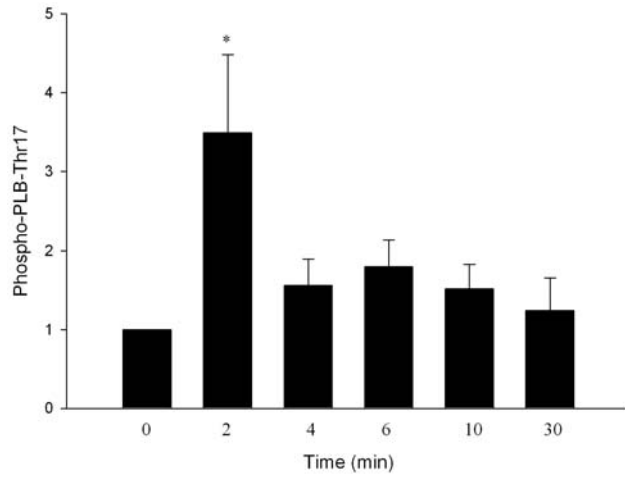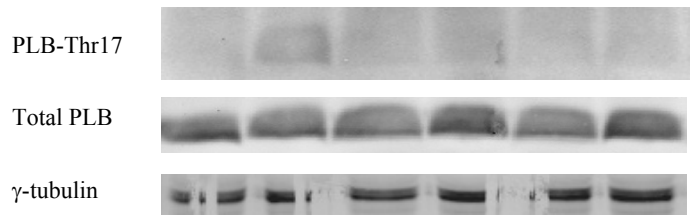
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Figure 2.4 continued…

E

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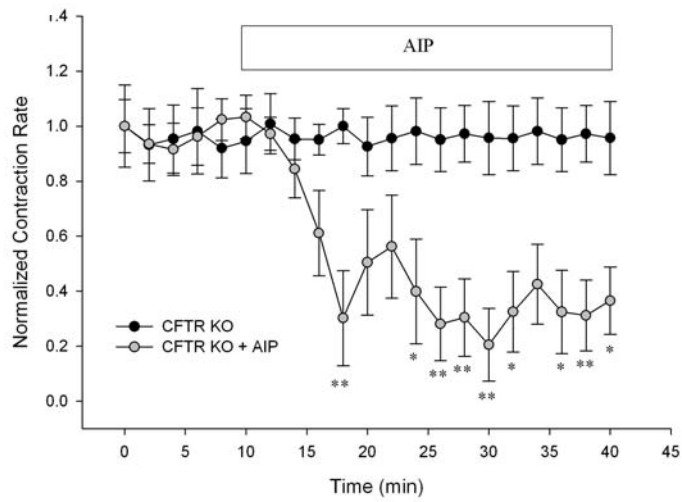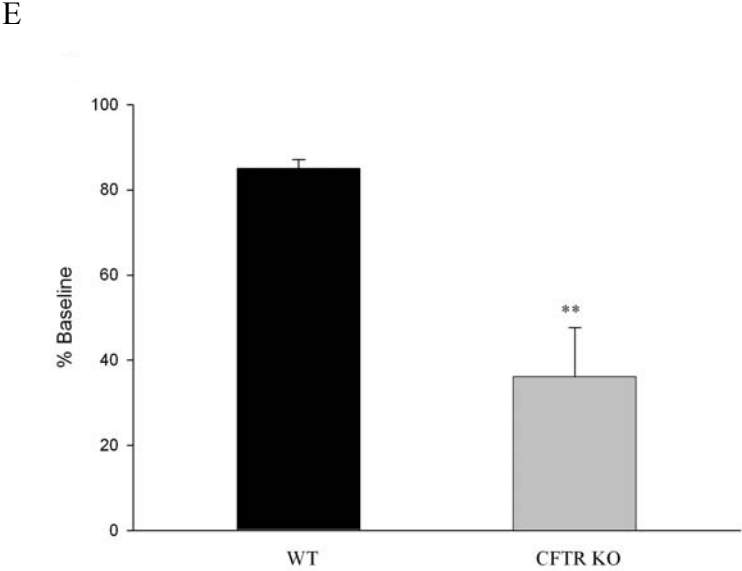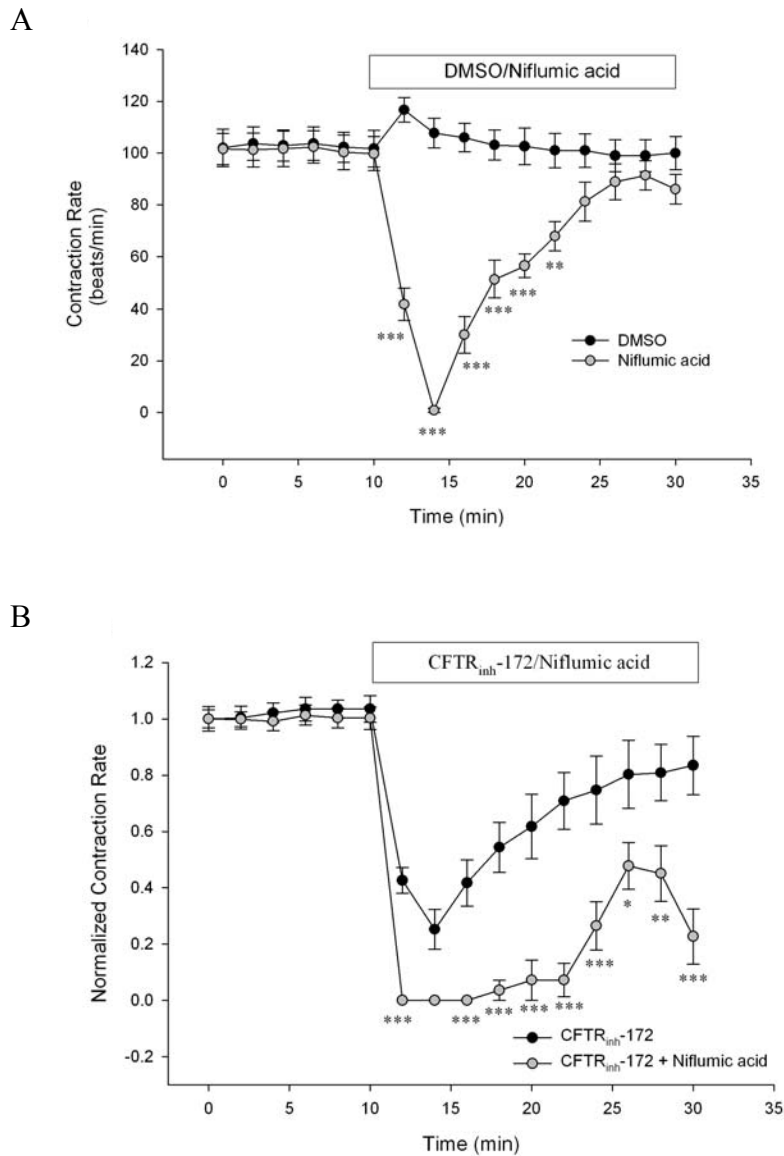
Figure 2.5. CaCC play an important role in cardiomyocyte contraction rate following CFTR inhibition or KO. A. Syncytial WT ventricular myocytes were treated with DMSO (n = 6) or niflumic acid (100 μM, n = 7) for 30 minutes following a 10-minute baseline. **B.** Separately, the effect of simultaneous addition of niflumic acid (100 μM) plus CFTR$_{inh}$-172 (20 μM, n = 8) for 20 minutes was examined and compared to the effect of CFTR$_{inh}$-172 alone (from Figure 1A), **C.** Ventricular myocytes from CFTR KO mice (n = 8) were treated with niflumic acid (100 μM) for 20 minutes after baseline measurements. Contraction rates were compared to those from CFTR KO mice in Figure 1A. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* CFTR KO alone by two-way ANOVA. **D.** A comparison of changes in contraction rate due to niflumic acid in WT and CFTR KO mice. ***, $P < 0.001$ *vs.* WT by student's t-test.

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Figure 2.5 continued…

C

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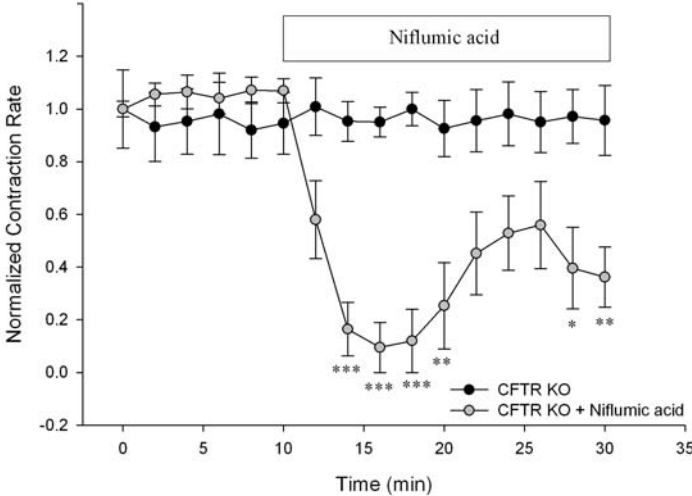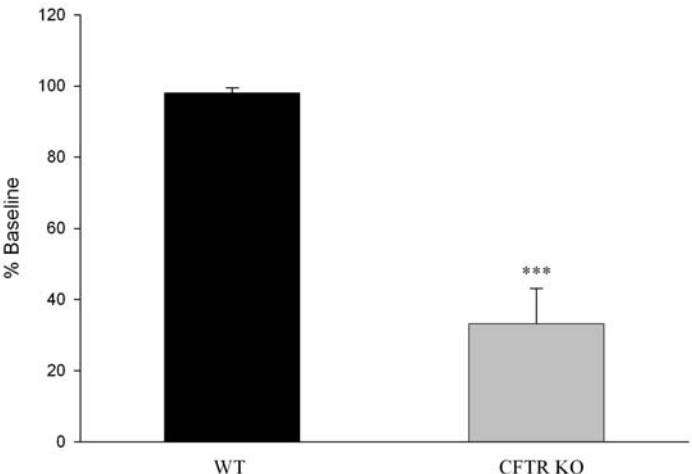
CHAPTER 3:

MULTI-DRUG RESISTANT PROTEIN 4 IN CARDIAC MYOCYTES: ANOTHER PLAYER IN THE REGULATION OF INTRACELLULAR cAMP AND β-ADRENERGIC SIGNALING

ABSTRACT

Spatiotemporal regulation of cAMP in cardiac myocytes is integral to regulating the diverse functions downstream of β-adrenergic stimulation. Phosphodiesterases (PDEs) have shown to be important in regulating intracellular cAMP levels during β-adrenergic stimulation. Recently, in epithelial and smooth muscle cells, it was found that the multidrug resistant protein 4 (MRP4) acts as a cAMP efflux pump to regulate intracellular cAMP levels and alter effector function. In the current study we investigated the potential role of MRP4 in regulating intracellular cAMP and β-adrenergic stimulated contraction rate in cardiac myocytes. Cultured neonatal ventricular myocytes were used for all experiments. In addition to wildtype mice, β_1-, β_2-, β_1/β_2-adrenergic receptor, and cystic fibrosis transmembrane conductance regulator (CFTR) knockout mice were used. MRP4 expression was probed via Western blot, intracellular cAMP was measured by fluorescent resonance energy transfer, while the functional role of MRP4 was assayed via monitoring of isoproteronol-stimulated contraction rate. We found that MRP4 is expressed in mouse neonatal ventricular myocytes. A specific pharmacological inhibitor of MRP4, MK571, resulted in accumulation of intracellular cAMP and potentiated submaximal isoproteronol stimulation via β_1-adrenergic receptors. Additionally, this potentiating effect on contraction rate was dependent on CFTR expression. This was in contrast to PDE4-dependent potentiation of contraction rate, which was not CFTR-dependent. Thus, we have shown, for the

first time, a role for MRP4 in the regulation of cAMP and β-adrenergic signaling in cardiac myocytes. Together with PDEs, MRP4 must also be considered when examining cAMP regulation in cardiac myocytes.

INTRODUCTION

The spatial and temporal control of cAMP has become widely accepted as the means whereby a single signaling molecule can lead to a diverse array of cellular responses. This concept has been widely studied in cardiac myocytes and recently commented on (1). Multi-layer compartmentalization of cAMP signaling occurs as a result of differential localization of β-adrenergic receptor subtypes, adenylyl cyclase, PKA, Epac, and phosphodiesterases (PDEs) via anchoring proteins, such as A-kinase anchoring proteins (2). The concentration and spread of cAMP is considered to be largely controlled by PDEs, which are responsible for terminating the signal by breakdown of cAMP. In cardiac myocytes β-adrenergic-stimulated increases in cAMP are predominantly regulated by PDE4 with PDE3 playing a minor role (3-5).

Multidrug resistance protein 4 (MRP4) is a member of the ATP-binding cassette (ABC) transporters, and was originally described in T-lymphoid cell line as being responsible for efflux of antiviral nucleoside-based drugs (6), it has since been found to transport numerous endogenous molecules involved in cellular communication and signaling, including, but not limited to, organic anions, prostaglandins, conjugated steroids, and cyclic nucleotides (7). Of particular interest, MRP4 has recently been shown to negatively regulate intracellular cAMP levels in both epithelial and smooth muscle cells (8, 9). In colonic epithelial cells, Li *et al* showed that inhibition of MRP4 by MK571 caused a compartmentalized increase in cAMP that resulted in activation of the cystic fibrosis transmembrane conductance regulator (CFTR) via

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PDZK1, which serves as a scaffolding protein for MRP4 and CFTR (8). In addition to colonic

epithelial cells, MRP4 is expressed in prostate tubuloacinar cells, hepatocytes, choroids plexus

epithelium, testis, ovary, adrenal gland, various blood cells, and neurons (7, 10); however, no

one has examined its presence in cardiac myocytes.

MATERIALS AND METHODS

Ventricular myocytes were cultured from wildtype (FV/B), β-adrenergic knockout (β_1,

β_2, β_1/β_2 KO), or CFTR KO (strain 2515 B6.129S6-$Cftr^{tm1Kth}$/J) neonatal mice were used for all

experiments. Cells were isolated and cultured using a similar protocol as previously reported

(11). Cells were plated at high-density for measurement of contraction rate or low-density for

cAMP measurements. High-density plating allowed for cells to form a syncytium, whereas low-

density plating facilitated individual quiescent myocytes.

For expression studies, cultured cardiomyocytes were isolated in 1X dulbecco's

phosphate buffer solution containing the protease inhibitors PMSF (250 μM), NaF (2.5 mM),

Na_3VO_4 (2.5 mM), benzamidine (1 mM) and lysed in PBS with 2% Triton-X 100 and

centrifuged prior to protein estimation. For electorphoresis, samples were run out on a 4-15%

agarose gel. Following transfer to a PVDF membrane, MRP4 was detected using an IgG anti-

MRP4 antibody.

We utilized fluorescent resonance energy transfer (FRET) to measure real-time changes

in intracellular cAMP. 24 hours after initial culture, isolated cardiomyocytes were infected with

adenovirus containing the cAMP sensor ICUE3 for 24-48 hours. Acquisition was set to 200

millisecond exposure in both channels every 20 seconds. The binding of cAMP to the EPAC site

of ICUE3 causes a decrease in FRET (12). Yellow-to-cyan ratios were calculated at different time points, background corrected, and normalized.

Measurement of myoctye contraction rate was performed as previously reported (13). 35 mm dishes were placed in a temperature/CO_2 regulated apparatus. Using a CCD camera and MetaMorph imaging software, 5-second video images were obtained every 2 minutes. Movements in the x and y plane were calculated and number of deflections noted as number of contractions.

Isoproterenol came from Sigma-Aldrich (St. Louis, MO). MK571 was purchased from Cayman Chemical (Ann Arbor, MI). Rolipram was from Calbiochem (San Diego, CA). $CFTR_{inh}$-172 was obtained from Calbiochem and the Cystic Fibrosis Foundation Therapeutics (Chicago, IL). Drugs were made as 1:1000 stock solutions and diluted the day of the experiment.

For comparison between two groups of data, student's t-test was used to determine significance, while multiple groups were compared by analysis of variance (ANOVA). Results were considered significant at $P < 0.05$ and are denoted accordingly.

RESULTS AND DISCUSSION

We first examined the expression of MRP4 in mouse neonatal ventricular myocytes. As seen in Figure 3.1A, in two different samples, a specific anti-MRP4 antibody recognized MRP4 protein. Subsequently, we examined if MRP4 was functional and if it regulated intracellular cAMP, as has been shown in other cell types (8, 9). We first examined the effect of MK571 (40 μM), a specific inhibitor of MRP4, on resting intracellular cAMP. While DMSO had a short-lived artifactual effect on cAMP measurements, it had no lasting effect on intracellular cAMP.

In contrast, MK571 caused a significant and prolonged increase in resting cAMP levels (Figure 3.1B). Thus we see that MRP4 is involved in regulating intracellular cAMP levels during resting conditions. While significant increases in resting cAMP can be achieved through broad inhibition of PDEs by agents such as IBMX, individual inhibition of PDE3 or PDE4, which constitute the major types in the heart, produce zero to minimal effects on resting cAMP (14). Therefore, with the substantial elevation of cAMP by MRP4 inhibition, we propose that MRP4 is a major regulator of resting cAMP.

β-adrenergic receptor activation, and subsequent cAMP generation, is a physiological event well-utilized by the body to manipulate cardiac function in response to various stimuli. Therefore, we examined the potential role of MRP4 in modulating β-adrenergic stimulated cAMP generation. Similar to previous findings (Soto *et al*, in preparation), in DMSO-treated cells, stimulation with submaximal isoproterenol (10^{-8} M) resulted in a transient increase in cAMP (Figure 3.1C), whereas maximal (10^{-5} M isoproterenol) stimulation caused a prolonged increase in intracellular cAMP (Figure 3.1D). In contrast, simultaneous addition of MK571 and submaximal isoproterenol led to a rapid and prolonged increase in cAMP that was significantly different from DMSO and isoproterenol stimulation ($P < 0.001$) (Figure 3.1C). Stimulation with MK571 and maximal isoproterenol led to a similar response ($P > 0.001$ *vs.* DMSO and submaximal) and overall was no longer significantly different than stimulation with DMSO and maximal isoproterenol; although some initial time points following stimulation were statistically increased ($P < 0.05$). In the previously mentioned study by Soto *et al*, they found that PDE4 inhibition led to similar increases in cAMP as we observed for MRP4 inhibition. Based on these results and others (15), it has been proposed that activation of PDE4 following β-adrenergic receptor stimulation is responsible for the transient nature of submaximal stimulation. While it is

tempting to say that MRP4 inhibition is also involved in this process, we did not measure MRP4 activity during receptor stimulation. Future studies are necessary to determine if MRP4 activity is altered during β-adrenergic receptor stimulation and how this correlates with PDE4 and other activated/inhibited factors.

Subsequently, we examined if alteration of MRP4 activity resulted in a functional effect on cardiac myocytes through modulating β-adrenergic stimulated cardiomyocyte contraction rate. Compared to DMSO-treated cells, MK571 significantly increased cardiomyocyte contraction rate in response to stimulation by submaximal isoproteronol; whereas at maximal concentrations there was no difference between DMSO and MK571-treated cells (Figure 3.2A). These findings are in agreement with our findings that MK571 increases intracellular cAMP compared to DMSO at submaximal, but not maximal, concentrations of isoproterenol (Figure 3.1C and 3.1D). While we did not examine discrete microdomains, ratio images suggest that MK571 preferentially increases isoproterenol-stimulated cAMP at the plasma membrane. Additionally, we investigated if MRP4 potentiates submaximal isoproteronol-stimulated contraction rate in a non-specific manner or if it is receptor subtype specific. To do so we examined the effect of MK571 on submaximal isoproteronol stimulated potentiation of contraction rate in β_1, β_2, and β_1/β_2 KO mice. We found that MK571 caused a significant increase in submaximal isoproteronol stimulated contraction rate compared to control in β_2 KO mice, but not β_1 or β_1/β_2 KO mice (Figure 3.2B), indicating that MRP4 potentiates isoproteronol-stimulated contraction rate in a receptor-specific manner involving β_1-adrenergic receptors. These findings are in contrast to previous work showing that inhibition of PDEs can increase contraction rate in both β_1 and β_2 KO mice (15).

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Based on previous findings that MRP4 activity can influence CFTR activity in epithelial cells (8), we examined if MRP4-dependent potentiation of contraction rate involved CFTR. In repeating similar experiments above using CFTR KO mice, we found that CFTR was required for MK571-dependent potentiation of contraction rate (Figure 3.2C), indicating that CFTR is crucial to this process. To determine if PDE4-dependent potentiation of contraction rate by is also CFTR-dependent, we examined the effect of rolipram in CFTR KO mice. In contrast to our findings with MRP4, inhibition of PDE4 does not require CFTR as rolipram continued to increase contraction rate in response to submaximal isoproterenol stimulation (Figure 3.1D). Thus, while inhibition of both MRP4 and PDE4 increase cAMP and contraction rates during submaximal β-adrenergic stimulation, we have shown that they possess different properties in how they do so. MRP4 inhibition has been shown to increase cAMP in a highly localized manner (8), whereas it is thought that PDE4 inhibition may lead to increased diffusion of cAMP (3). Our observations that MK571 increased isoproterenol-stimulated cAMP along the plasma membrane, coupled with our findings that MRP4-dependent potentiation is CFTR-dependent, leads us to propose that in cardiac myocytes inhibition of MRP4 produces a an increase in cAMP near the membrane where CFTR is located. Whether MRP4 and CFTR physically interact in cardiac myocytes remains to be seen. Future experiments investigating the intricacies of how MRP4 activity is altered during β-adrenergic stimulation is required, however, we have shown for the first time that MRP4 is expressed and functional in cardiac myocytes and potentiates β-adrenergic stimulated contraction in a manner distinct from PDE4. Thus, future studies examining cAMP regulation in cardiac myocytes should take into consideration the potential involvement of MRP4.

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FIGURES

A

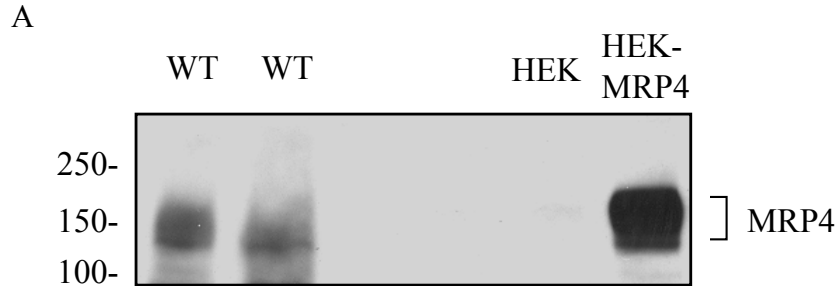**Figure 3.1. Expression and function of MRP4 in mouse neonatal ventricular cardiomyocytes. A.** Cardiomyocytes from two different samples (both WT) were cultured and harvested in lysis buffer. 200 μg and 40 μg of cardiomyocytes or HEK cells, respectively, were loaded into agarose gels. Expression of MRP4 was probed via an anti-MRP4 antibody (1:5000). **B.** WT ventricular myocytes infected with ICUE3 were exposed to DMSO (n ≥ 20) or MK571 (40 μM, n ≥ 22) for 20 minutes following a 3-minute baseline. **C and D.** In separate experiments, following baseline measurements, cardiomyocytes were stimulated with with DMSO + 10^{-8} M isoproterenol (n = 20) or MK571 (40 μM) + 10^{-8} M isoproterenol (n = 23) for 20 minutes. Similar experiments were also carried out with 10^{-5} M isoproterenol (DMSO, n = 21; MK571, n = 25). Representative ratios of cardiomyocytes are shown in pseudocolor at baseline and peak responses for each treatment.
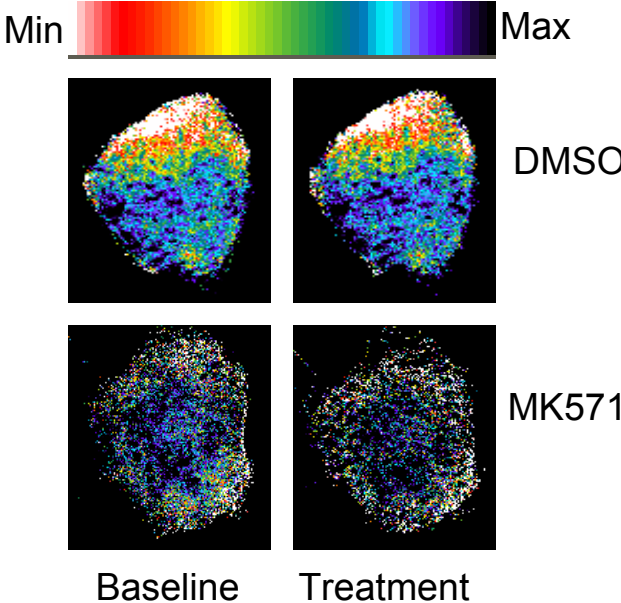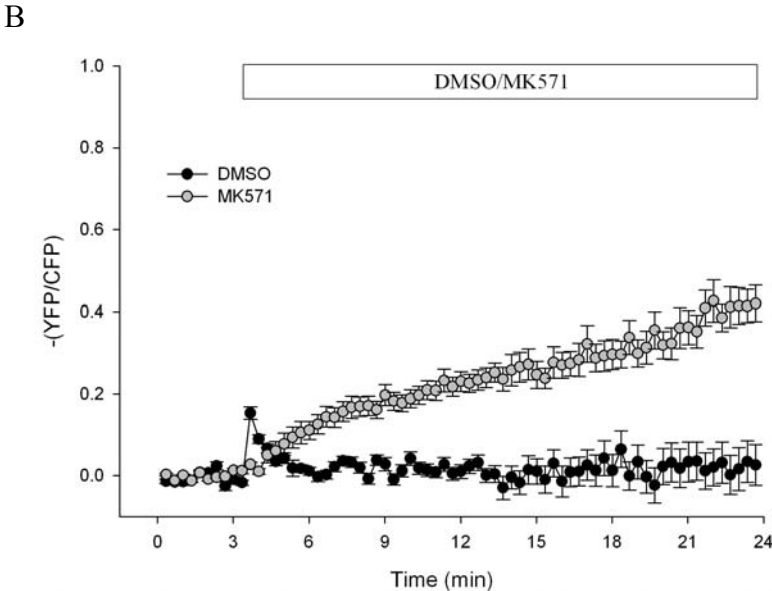
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B

Figure 3.1 continued…

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DMSO

MK571

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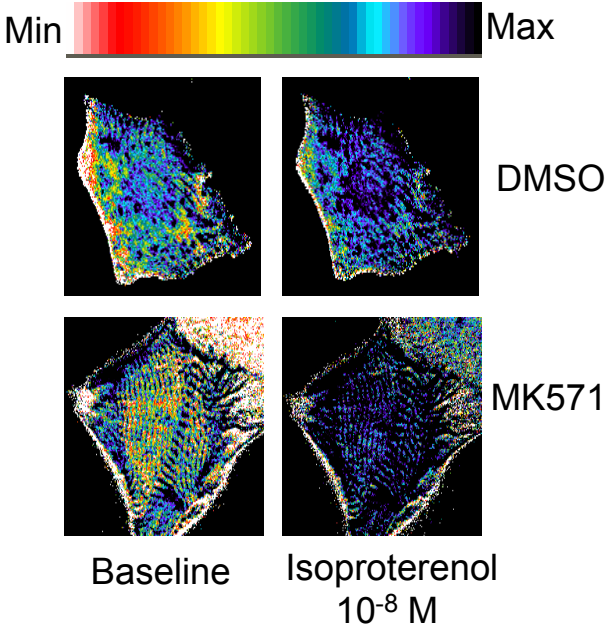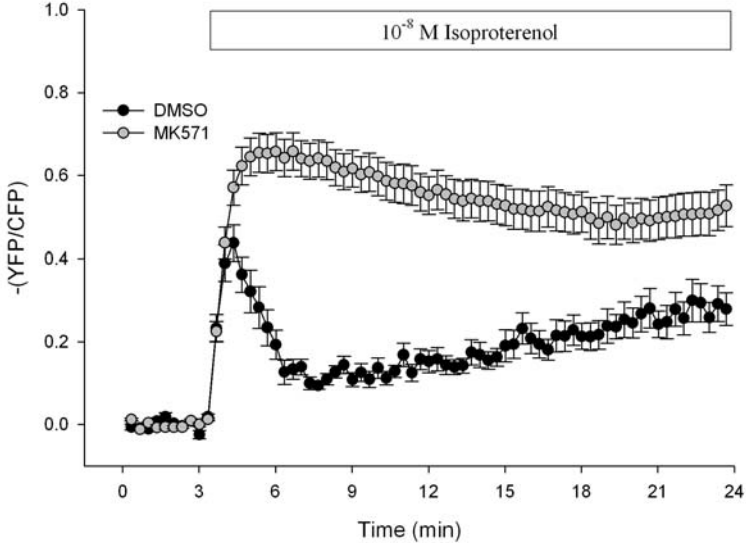
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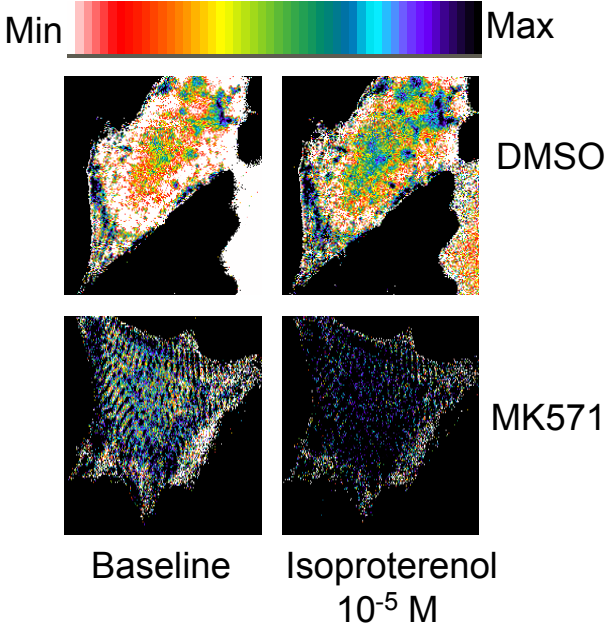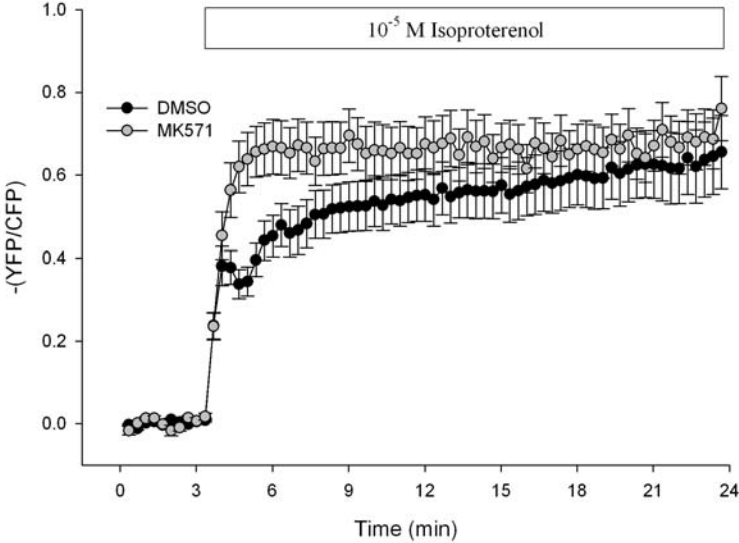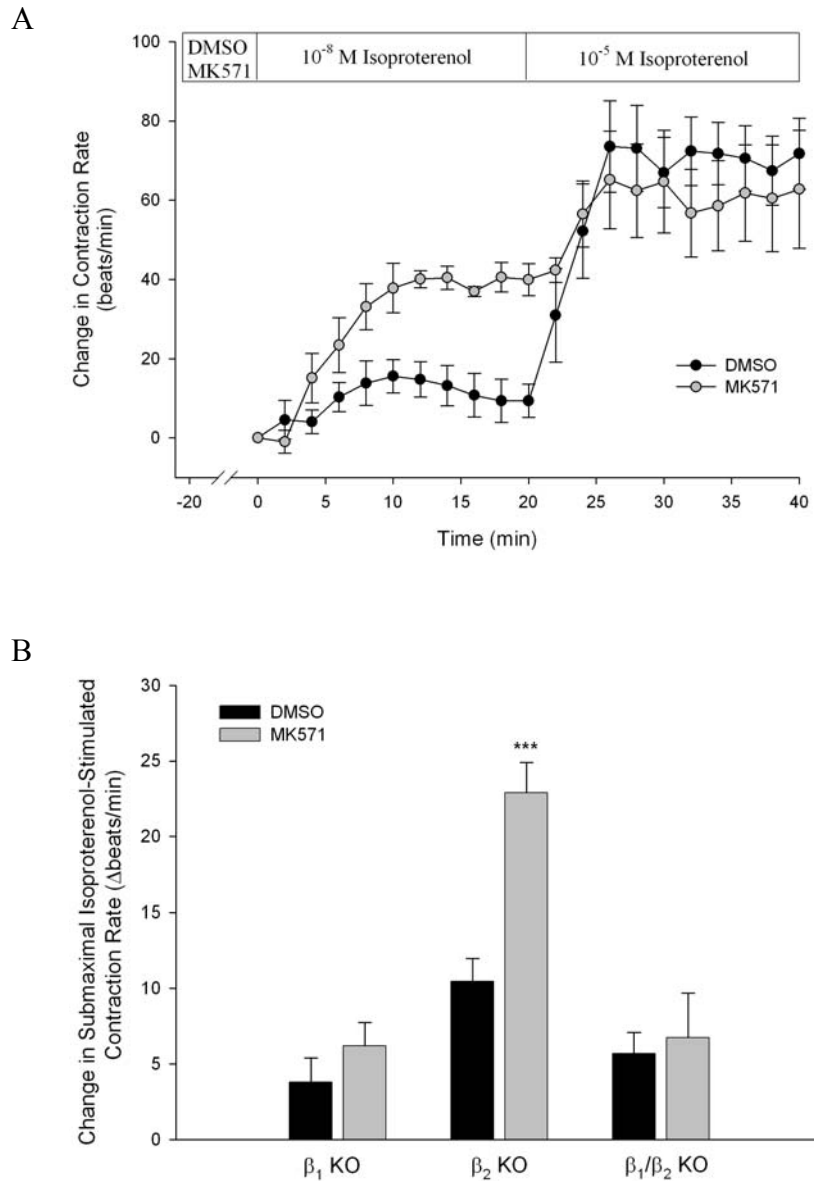
Figure 3.2. Characterization of the effect of MRP4 inhibition on cardiomyocyte contraction rate. A. Spontaneous beating syncytial WT ventricular myocytes were pre-treated with DMSO (n = 5) or MK571 (40 μM, n = 5) for 20 minutes prior to isoproterenol stimulation. Cardiomyocytes were stimulated with submaximal isoproterenol (10^{-8} M), followed by maximal isoproterenol (10^{-5} M), for 20 minutes. **B.** Additionally, the effects of MK571 on submaximal isoproterenol stimulation was examined in β_1 KO, β_2 KO, β_1/β_2 KO mice (n $\geq$ 6). **C.** CFTR KO mice (n = 6) were exposed to MK571 in a similar manner as Figure 2A. **D.** Additionally, in separate experiments, the same types of conditions were tested using rolipram (10 μM; n $\geq$ 6) instead of MK571. ***, P<0.001 *vs.* β_2 KO DMSO by student's t-test.

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Figure 3.2 continued…

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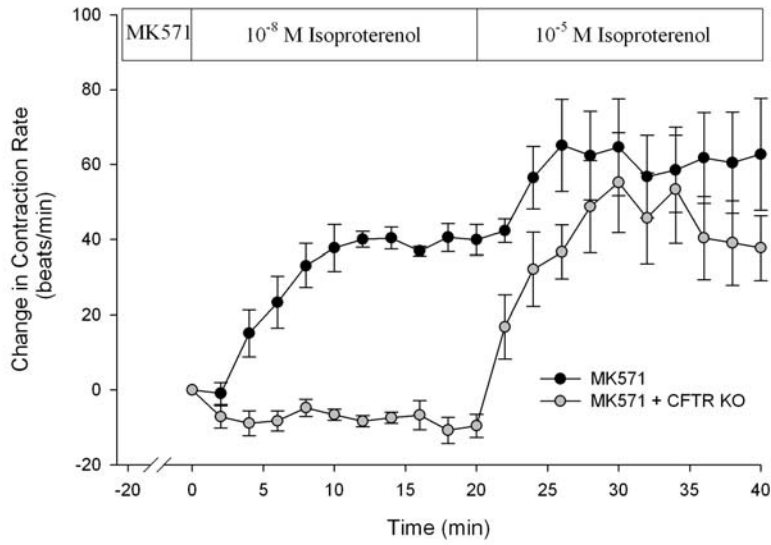
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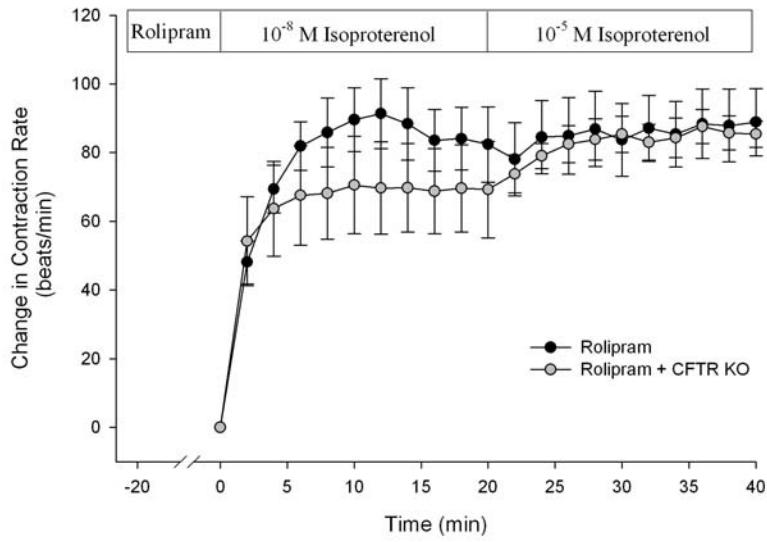
CHAPTER 4:

IN VITRO AND *IN VIVO* EXAMINATION OF CFTR IN β-ADRENERGIC STIMULATED HEART FUNCTION

ABSTRACT

β-adrenergic receptor stimulation triggers a diverse array of intracellular signals that affects many ion transporters and channels in an effort to increase cardiac output. With our previous findings that CFTR is involved in the regulation of un-stimulated cardiomyocyte contraction rate, we set out to determine if CFTR is also involved in β-adrenergic stimulated contraction rate. To do this, we utilized *in vitro* and *in vivo* approaches using the neonatal cardiomyocyte contraction rate assay and left ventricular catheterization to measure heart rate, systolic, and diastolic function. Post-mortem structural analysis and immunohistochemistry was also performed. *In vitro*, we found that positive or negative regulation of CFTR activity using the CFTR activators genistein and PG-01 or the CFTR inhibitor $CFTR_{inh}$-172 and CFTR knockout myocytes preferentially affect submaximal isoproterenol-stimulated contraction rate in a positive or negative manner, respectively. *In vivo*, CFTR knockout mice displayed altered resting cardiac function parameters with increased heart size, heart rate, contraction, and relaxation, while maintaining normal left ventricular end-diastolic pressure. β-adrenergic stimulation with dobutamine produced normal increases in heart rate, contraction, and relaxation, however, at the expense of increased left ventricular end-diastolic pressure. Structural disturbances preferentially affected male knockout mice and may correlate with decreased Ca^{2+}/calmodulin-dependent kinase II expression in male CFTR knockout mice. Our findings show that CFTR is involved in the modulation of β-adrenergic stimulated heart function and that

chronic loss of CFTR leads to cardiac disturbances. These findings have important implications

for cystic fibrosis patients and their potential susceptibility to heart disease.

INTRODUCTION

Proper inotropic control of the heart is integral to maintaining heart function, as

illustrated by the loss of inotropy during heart failure and the widespread clinical use of positive

inotropic agents to combat the ill effects of heart failure. Tight control of cardiomyocyte

inotropy occurs predominantly through β-adrenergic receptors and their diverse downstream

effectors. Activation of different β-adrenergic receptors (β_1 *vs*. β_2 *vs*. β_3) produces distinct

intracellular signals and functional responses in cardiomyocytes (1). In particular, β_1-adrenergic

receptor activation has been shown to be the primary mode of increasing cardiac output by

increasing the contraction rate and contractility of cardiac muscle cells. Simplistically, β_1-

receptor activation leads to activation of protein kinase A (PKA) via increased production of

cAMP by adenylate cyclase. Subsequently, PKA directly phosphorylates and enhances the

activity of a host of ion channels and transporters, which have been summarized by Kuzumoto *et*

al (2), but include the sarcolemmal L-type Ca^{2+} current, the slowly activating component of

delayed rectifier K^+ current, the plasma membrane Ca^{2+}-ATPase, the ryanodine receptor (3), and

pospholemman and phospolamban, which regulate the Na^+/K^+ ATPase and sarcoplasmic

reticulum Ca^{2+} pump (SERCA), respectively (4). In addition, the cystic fibrosis transmembrane

conductance regulator (CFTR) anion channel is directly regulated by PKA phosphorylation of its

regulatory domain, leading to an increase in membrane Cl^- conductance.

Cardiac expression of CFTR has been shown in a variety of species, including rodents

and humans (5, 6). Simulation studies by Kuzumoto *et al* predicted that increases or decreases in

CFTR channel density would positively or negatively alter action potential duration, respectively, due to β-adrenergic stimulation (2). However, in electrophysiological studies there has been controversy whether CFTR in the heart is activated by β-adrenergic stimulation, with Zakharov *et al* reporting isoprenaline-stimulated CFTR currents (7), and Levesque *et al* reporting that isoprenaline failed to stimulate CFTR currents (8). These conflicting reports have led to questions regarding the importance of CFTR in the heart. However, in non-electrophysiological experiments, CFTR has been shown to be up-regulated during ischemia (9), involved in the protection of ischemic preconditioning (10), and down-regulated in patients with heart failure (11). Additionally, we have shown that in neonatal ventricular myocytes CFTR is involved in regulating contraction rate during un-stimulated spontaneous beating (Chapter 2).

The ongoing question of the importance of CFTR in cardiac physiology has also been confounded by the complexity of studying its effects in humans. In other systems, cystic fibrosis (CF) patients, who have defects in CFTR, provide the ideal model in defining the role of CFTR. However, these patients typically present with significant lung disease, which can indirectly lead to alterations in left ventricular function through right ventricular defects. Thus, studies that have reported left ventricular defects in CF patients are confounded by a clear understanding of whether these CFTR defects in the heart lead to primary disruptions in cardiac function or are secondary to lung function abnormalities. To address these issues and understand if CFTR is physiologically involved in β-adrenergic stimulation heart function we undertook a series of *in vitro* and *in vivo* experiments utilizing acute pharmacological inhibition of CFTR and chronic deletion of CFTR with CFTR KO mice. These methods allowed us to examine potential primary defects in cardiac myocytes and since CFTR KO mice do not develop lung disease, we can study heart function *in vivo* without the complications of lung pathology. *In vitro*, we found that loss

of CFTR leads to primary defects in β-adrenergic stimulated cardiomyocyte contraction. CFTR KO mice showed altered dependence on other ion channels, such as Ca^{2+}-activated Cl^- channels, which allows for compensation under some, but not all, circumstances. *In vivo*, we found heart function abnormalities under un-stimulated and stimulated conditions. Additionally, post-mortem analysis of CFTR KO hearts, revealed structural changes and alterations in CaMKII expression. These findings may have implications for CF patients in understanding their risks for heart disease.

MATERIALS AND METHODS

Ethical approval of animal use. FV/B mice were obtained from Charles River Laboratory (Wilmington, MA) and used for generation of wildtype (WT) mice. CFTR KO mice (strain 2515 B6.129S6-*Cftr^{tm1Kth}*/J) were initially obtained from Dr. Deborah Nelson at the University of Chicago (Chicago, IL) and bred in-house. Neonatal mice were were used within 24 hours of birth while adult animals were 2-4 months old. Use of animals was approved by the University of Illinois at Urbana-Champaign IACUC.

Isolation and culture of neonatal and adult mouse cardiomyocytes. Ventricular myocytes were cultured from neonatal and adult mice for using a similar protocols as previously reported (12). For neonatal cardiomyocytes, freshly born mice were sacrificed by decapitation with surgical scissors. Cardiac tissue devoid of atria were exposed to enzymatic digestion. Subsequently, isolated cells were pipetted onto 35 mm Petri dishes pre-coated with 1.5% gelatin type A and plated at high-density to facilitate formation of a syncytium of spontaneously beating myocytes. Cells were cultured fro 2-3 days and culture media (containing serum and 20 mM HEPES for buffering) was replaced daily and at least 1 hour prior to experiments.

Measurement of contraction rate *in vitro*.   Measurement of neonatal myoctye contraction rate was performed as previously reported (13).   35 mm dishes were placed in a temperature/CO_2 regulated apparatus. Using a CCD camera and MetaMorph imaging software, 5-second video images were obtained every 2 minutes. Following acquisition, a specific point within the field was selected and analyzed for movement in each x, y, and z plane. Movements in the x and y plane were calculated and number of deflections noted as number of contractions. Each data point represents a single observation from different experiments so that only one data set was obtained from each experiment. Each drug was tested using multiple isolations to ensure that the observed responses were not particular to a single isolation.

Measurement of heart function *in vivo*. The protocol for in vivo hemodynamic studies in the mouse was derived from the description by Rockman *et al* (14) and carried out by the Mouse Cardiovascular Phenotyping Core Facility at Washington University, St. Louis. Adult mice were anesthetized with thiopental sodium (60 mg/kg, intraperitoneally). This anesthesia produces a normal heart rate of 500 beats/min while still allowing for a surgical plane of anesthesia. The mice are then intubated and ventilated with a Harvard ventilator set at 200-400 μL. The bilateral carotid arteries were identified in the region of the trachea and cannulated with a 1.4 french high fidelity micromonometer catheter from Millar Instruments, which was then inserted into the cartotid artery, advanced across the aortic valve, and secured in the left ventricle. Hemodynamic measurements were recorded at baseline and 60 seconds after injection of bolus doses of dobutamine. Continuous left ventricular (LV) systolic and diastolic pressures, and the derivatives of left ventricular pressure (dP/dt) were recorded on a laptop commuter with Millar analysis software. After successful recording of functional data, mice were euthanized by anesthetizing with thiopental sodium followed by cervical dislocation.

Histology. Brains, spleens, and hearts were promptly removed from euthanized adult mice and placed in cassettes in 10% formalin for 24 hours. Susbseqently, tissues were dried in 70% ethanol and shipped to the Histology Core Facility at the University of California, San Diego. There tissues were paraffin embedded, sectioned, and processed for hematoxylin and eosin (H&E), TUNEL, trichrome, periodic acid-schiff (PAS), and immunohistochemistry with antigen retrieval using established protocols. Ca^{2+}/calmodulin-dependent kinase II (CaMKII) was detected using a polyclonal rabbit anti-CaMKII antibody from Santa Cruz Biotechnology (H300). Spleens were used as controls for proliferation and brains as controls for CaMKII expression. Tissues were coded so that their identities were blinded to the technicians and histopathologist (Dr. Nissi Varki, UCSD) working with and analyzing samples. Additionally, using H&E sections, the areas of left and right ventricles and interventricular septum were measured using ImageJ software.

Chemicals. Isoproterenol and genistein were purchased from Sigma-Aldrich (St. Louis, Missouri), $CFTR_{inh}$-172 and PG-01 was obtained from Cystic Fibrosis Foundation Therapeutics (Chicago, IL). $CFTR_{inh}$-172 was also purchased from Calbiochem (San Diego, CA). The Mouse Cardiovascular Phenotyping Core Facility at Washington University, St. Louis, independently acquired dobutamine.

Statistics. All data are expressed as means ± SEM, except where indicated. Student's t-test was used for comparison of two groups, two-way ANOVA was used to compare time courses and one-way ANOVA was used to compare multiple groups. Statistics were performed by GraphPad Prism software, while EC_{50}s were calculated using SigmaPlot.

RESULTS

Measurement of *in vitro* cardiomyocyte contraction rate. To determine if CFTR has an intrinsic role in β-adrenergic stimulated heart function, we examined the effect of acute and chronic CFTR loss on isoproterenol-stimulated contraction rate. In WT cardiomyocytes isoproterenol dose-dependently increased cardiomyocyte contraction rate (Figure 4.1). In contrast, in WT myocytes pretreated with the selective CFTR inhibitor, $CFTR_{inh}$-172 (20 μM), or CFTR KO mice, isoproterenol failed to stimulate any increase in contraction rate at submaximal concentrations (Figures 4.1.A-C). Specifically, while 10^{-8} M isoproterenol stimulated a significant increase in contraction rate in control myocytes, this same concentration produced no response in $CFTR_{inh}$-172-treated myocytes or those from CFTR KO mice. Despite these defects, CFTR loss had no deleterious impact on concentrations of isoproterenol that stimulated maximal increases in contraction rate (10^{-7} M to 10^{-5} M). To further understand the impact of CFTR activity on contraction rate, we performed similar experiments with myocytes pretreated with genistein and PG-01, which have been shown to directly activate CFTR (15, 16). In a similar manner as CFTR loss, increasing the activity of CFTR preferentially affected contraction rates at submaximal isoproterenol stimulation (10^{-8} M), with no effect on maximal stimulation (10^{-5} M) (Figure 4.1.C). Thus, modulation of CFTR activity appears to preferentially modulate submaximal isoproterenol stimulated contraction rate, with gain of CFTR activity leading to a right-shift and loss of CFTR activity a left-shift of the isoproterenol-stimulated contraction rate dose response curve (Table 4.1).

In lung and intestinal epithelial cells CFTR has been shown to associate with $β_2$-adrenergic receptors (17, 18). Therefore, we sought to determine if CFTR-dependent depression of contraction rate involves $β_2$-adrenergic receptors. Utilizing β-adrenergic receptor KO mice,

we found isoproterenol stimulated contraction rate increases at both submaximal (10^{-8} M) and maximal (10^{-5} M) doses (Figure 4.2A) in a manner that was dependent on β_1-adrenergic receptors. Subsequently, using β_2 KO mice, we repeated previous experiments examining the effect of CFTR inhibition on submaximal and maximal stimulated contraction rate using CFTR$_{inh}$-172. In β_2 KO mice, CFTR$_{inh}$-172 (20 μM) continued to preferentially inhibit submaximal isoproterenol (10^{-8} M) stimulated contraction rate, with no effect at maximal stimulation (10^{-5} M) (Figure 4.2B). Thus, in cardiac myocytes CFTR appears to modulate β_1-adrenergic receptor stimulation to control increases in contraction rate.

In an effort to understand why CFTR is particularly critical for submaximal β-adrenergic stimulated contraction rate we examined the effect of PDE4 and MRP4 inhibition, which have been shown to modulate intracellular cAMP levels in cardiac myocytes (19)(Chapter 3), on contraction rate during CFTR loss. In cardiomyocytes with functional CFTR (control), inhibition of PDE4 or MRP4 by rolipram (10 μM) or MK571 (40 μM), respectively, led to a significant increase in submaximal isoproterenol (10^{-8} M) stimulated contraction rate (Figure 4.3). In WT myocytes pretreated with CFTR$_{inh}$-172 or myocytes from CFTR KO mice, rolipram continued to cause an increase in isoproterenol-stimulated contraction rate, although the magnitude between acute and chronic loss of CFTR differed (P < 0.001). In contrast, MK571 failed to stimulate any increase submaximal isoproterenol stimulated contraction rate in CFTR$_{inh}$-172 treated or CFTR KO cardiomyocytes. Thus, it appears that while CFTR is critical for submaximal stimulated increases in contraction rate under normal circumstances, this defect can be overcome by inhibition of PDE4, but not by MRP4.

Subsequently, we sought to determine if the normal increases in maximal stimulated contraction rate were a result of CFTR not being involved in the response to maximal stimulation

or if acute or chronic loss of CFTR led to altered regulation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and/or Ca^{2+}-activated Cl^- channels, as we have shown previously for baseline contraction (Chapter 2). To probe this we examined the effect of myristoylated-autocamtide-2-inhibitory peptide (AIP; 1 μM) or niflumic acid (100 μM) on maximal (10^{-5} M) isoproterenol-stimulated contraction rates in WT myocytes with and without CFTR inhibition and in CFTR KO mice. As seen in Figure 4.4A, inhibition of CaMKII by AIP caused a significant elevation in isoproterenol-stimulated contraction rate. In contrast, when WT myocytes were pretreated with AIP and $CFTR_{inh}$-172, isoproterenol (10^{-5} M) failed to potentiate contraction rate, but continued to increase contraction rate similar to control myocytes (Figures 4.4B-C). However, in the case of CFTR KO cardiomyocytes treated with AIP, isoproterenol potentiated contraction rate in a similar manner as AIP in WT myocytes (Figures 4.4B-C). Subsequently, we examined the effect of niflumic acid. In WT mice, we found that niflumic acid significantly attenuated isoproterenol-stimulated contraction rate, with more pronounce effects over time (Figures 4.5A, C), indicating that Ca^{2+}-activated Cl^- channels are important in contraction rate responses to maximal isoproterenol stimulation. When we examined how these responses were affected by acute and chronic loss of CFTR activity, we found that treatment of WT myocytes with niflumic acid and $CFTR_{inh}$-172 significantly inhibited any increase in isoproterenol-stimulated contraction rate (Figures 4.5B-C). In contrast, niflumic acid treatment in CFTR KO mice produced similar responses as niflumic acid treatment in WT mice (Figure 4.5B). Thus, we have shown that during acute, but not chronic, CFTR loss, Ca^{2+}-activated Cl^- channels appear to play an increased role in contraction rate increases due to maximal isoproterenol stimulation.

Measurement of *in vivo* heart function. Subsequently, to determine if loss of CFTR leads to *in vivo* cardiac disturbances, we measured *in vivo* left ventricular function in WT and

CFTR KO mice. Since CFTR KO mice do not display lung abnormalities, it is an ideal model for studying heart function independent of lung dysfunction. Through catheterization of the left ventricle we obtained measurements for heart rate, indices of contraction (+dP/dt) and relaxation (-dP/dt), and left ventricular end-diastolic pressure (LVEDP). As seen in Figure 4.6, in comparison to WT mice, CFTR KO mice display altered heart function at rest. We found that CFTR KO mice had significantly elevated heart rate, +dP/dt, and -dP/dt at rest, indicating that heart activity is increased in these mice (Figure 4.6A-C). While activity was increased, there did not appear to be any dysfunction as resting LVEDP in CFTR KO mice was normal compared to WT mice (Figure 4.6D). Following resting measurements, we perfused increasing concentrations of the inotropic agent dobutamine, which serves as a β-adrenergic agonist *in vivo* (20), and measured subsequent changes in heart function. In contrast to resting measurements, dobutamine stimulated similar increases in heart rate, +dP/dt, and -dP/dt compared to WT mice, however, CFTR KO mice developed significantly elevated LVEDP during stimulation (Figure 4.7). Thus, while CFTR KO mice are able to properly respond to stimulatory conditions, this comes at the cost of increased dysfunction as indicated by changes in LVEDP. Post-mortem analysis of heart sizes revealed a trend in an increased size of hearts from CFTR KO mice compared to WT mice (Figure 4.8A), further indicative of heart abnormalities in CFTR KO mice.

Characterization of heart structure. In addition to functional studies, in separate animals, we performed an in depth analysis of the structure of the hearts from WT and CFTR KO mice to determine if any abnormalities existed. H&E, PAS, Trichrome, and TUNEL assays revealed no significant differences between WT and CFTR KO mice, indicating no structural damage or elevated apoptosis in CFTR KO hearts. Despite these findings, measurement of left

ventricular (LV), right ventricular (RV), and interventricular septal sizes showed that CFTR KO mice have significantly elevated left ventricular and interventricular septal (IVS) areas (Figure 4.8B-C). Right ventricular size was comparable between WT and CFTR KO mice. Thus, this data indicates that CFTR KO mice, which have normal lung function (and corresponding normal right ventricle size), have elevated heart size due to increased left ventricular and interventricular septal areas.

CaMKII expression. Based on our findings that CaMKII inhibition can affect contraction rates (Figure 4.4, Chapter 2) and is involved in cardiac hypertrophy (21), we examined if CaMKII expression is altered in the hearts of CFTR KO mice. Immunohistochemical analysis showed that in 3 of 5 samples there was decreased CaMKII expression in CFTR KO mice, compared to WT mice (Figure 4.9). When we examined the origin of these tissues, we noticed that the tissues that showed decreased CaMKII expression were exclusively from male CFTR KO mice. When we partitioned the samples by gender we found that in all heart tissues from males CaMKII expression was decreased, whereas in female heart tissues there was no noticeable changes between WT and CFTR KO mice (Figure 4.9). Thus, it appears that chronic loss of CFTR leads to a down-regulation of CaMKII, however, this appears to be gender-specific, only occurring in males.

Potential gender differences. Based on our findings with CaMKII expression, we re-examined our heart structure data to determine if there were differences in male *vs.* female hearts. As seen in Table 4.2, separation of data by sexes shows that cardiac structural cardiac defects appear to predominantly affect male CFTR KO mice. Compared to WT mice, there was no difference between whole heart, left ventricle, or interventricular sizes in female CFTR KO

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mice. In contrast, the significance of these parameters in male CFTR KO mice was enhanced (see bolded boxes).

DISCUSSION

In the current study we provide the first experimental evidence that loss of CFTR causes primary defects in β-adrenergic stimulated heart function. In isolated cardiomyocytes we found that CFTR is critical for producing increases in submaximal β-adrenergic stimulated contraction rate. Interestingly, this defect could be overcome by inhibition of PDE4, but not MRP4. While both PDE4 and MRP4 negatively regulate intracellular cAMP (19, Chapter 3), PDE4 inhibition causes an increase in cytosolic cAMP, while MRP4 inhibition increases cAMP in a localized manner near the membrane (22)(Chapter 3). Thus, we propose that CFTR is responsible for responding to local changes in cAMP due to submaximal isoproterenol stimulation.

While CFTR loss alone does not alter responses to maximal β-adrenergic stimulation, this does not discount its involvement. When we inhibited both CFTR and Ca^{2+}-activated Cl^- channels we found that myocytes could no longer produce an increase in contraction rate, which was significantly different from Ca^{2+}-activated Cl^- channel inhibition alone. However, in CFTR KO mice niflumic acid produced the same response as in WT mice, indicating that other channels/transporters likely compensate during chronic CFTR loss. This may be explained by an up-regulation of repolarizing K^+ currents in CFTR KO mice, which would compensate for outward-conducting Cl^- current loss. Previously, Li *et al* found that chronic CaMKII inhibition led to an upregulation of the fast transient outward current and inward rectifier current (23). Our findings that CFTR KO mice show decreased expression of CaMKII provide a compelling

argument for future examination of the expression and activities of these K^+ channels in CFTR KO mice.

When we examined β-adrenergic stimulated heart function *in vivo* we did not find contraction defects at submaximal concentrations, as one might expect from our *in vitro* results. We cannot say whether this is due to a differential importance of CFTR in neonatal *vs.* adult myocytes or the increased activity of CFTR KO hearts during non-stimulated conditions. Since we did not measure circulating catecholamine levels in our mice, we cannot rule out a potential difference in underlying β-adrenergic stimulation. However, in our study we found that inhibition of CaMKII by AIP led to a significant increase in isoproterenol-stimulated contraction rates. If these results translate to adult mice, decreased expression of CaMKII in CFTR KO mice may be responsible for the increased heart activity we observed. Zhang *et al* found that chronic CaMKII inhibition led to an increase in Ca^{2+} transients, increased Ca^{2+}_i, decreased sarcoplasmic reticulum Ca^{2+}, and a trend in increased cardiomyocyte shortening (24); all of which could cause an increase in heart activity. Further examination of these properties in isolated CFTR KO cardiomyocytes will clarify whether the increased activity of CFTR KO hearts is due to direct alterations in cardiomyocyte properties or secondary to other factors.

Despite achieving normal β-adrenergic-stimulated increases in heart rate, contraction, and relaxation, CFTR KO mice developed an increase in LVEDP. With the absence of contraction/relaxation deficits, we hypothesize that this may be the result of increased afterload due to increased aortic constriction in CFTR KO mice (25). In future experiments, simultaneous examination of left ventricular function and aortic constriction will allow us to determine whether the increase in LVEDP in CFTR KO mice is due to cardiac or vascular defects. Regardless of the origin, defects in LVEDP due to CFTR loss are a significant concern as

increased LVEDP is a precursor to the development of heart disease. In fact, while hearts from CFTR KO mice did not show any signs of active apoptosis or inflammation, we did find structural alterations compared to WT mice. CFTR KO mice showed a trend in increased heart size compared to WT mice. When we microscopically examined the heart, right ventricular size was normal, as one would expect since these mice do not develop lung disease like CF patients. However, the interventricular septum and left ventricles of CFTR KO mice were enlarged, indicative of left ventricular hypertrophy. While CaMKII expression is positively associated with structural heart disease (21), hypertrophy can occur independent of CaMKII expression (26). Rather, this may be the result of chronic overstimulation of CFTR KO hearts or chronic aortic hyperconstriction. Future examination of the cause of this hypertophy is important in understanding how chronic CFTR loss affects the heart. Additionally, it is important to determine if CFTR KO hearts progress from hypertrophy (concentric hypertrophy) to atrophy and dilation (eccentric hypertrophy). Our current results suggest that decreased expression of CaMKII in CFTR KO mice may prevent the transition from concentric hypertrophy to eccentric hypertrophy (26), however, with increased heart activity and aortic constriction in CFTR KO mice, they may be at risk for heart failure through arrhythmogenesis and/or pressure overload.

Interestingly, we found that the structural changes occurring in CFTR KO mice predominantly affected male mice. Estrogens have been shown to activate CFTR (27, 28); however, it is unknown whether testosterone has any effect on CFTR activity. Since sex steroids differentially affect both heart function and cardiac disease progression (29), it will be necessary to decipher how testosterone and estrogens affect CFTR KO cardiovascular function. These findings may have direct impacts on gender-specific therapies for CF patients.

In summary, while further studies are required to clarify the effect of CFTR loss on cardiomyocytes and heart function, we have clearly shown that CFTR is involved in regulating heart function during β-adrenergic stimulation. These results, coupled with our previous results (Chapter 2) provide evidence that CFTR is involved in modulating both stimulated and un-stimulated cardiomyocyte contraction. While CFTR KO mice display "normal" cardiomyocyte contraction, this occurs at the expense of altered regulation of other ion channels, signaling mediators, stimulated heart function, and changes in heart structure. Thus, these results, in the context of all of our data, provide compelling evidence for the need to carefully investigate the heart structure and function of cystic fibrosis patients in order to determine their susceptibility to heart disease. Doing so may lead to an increase in both the quality and quantity of life experience by these patients.

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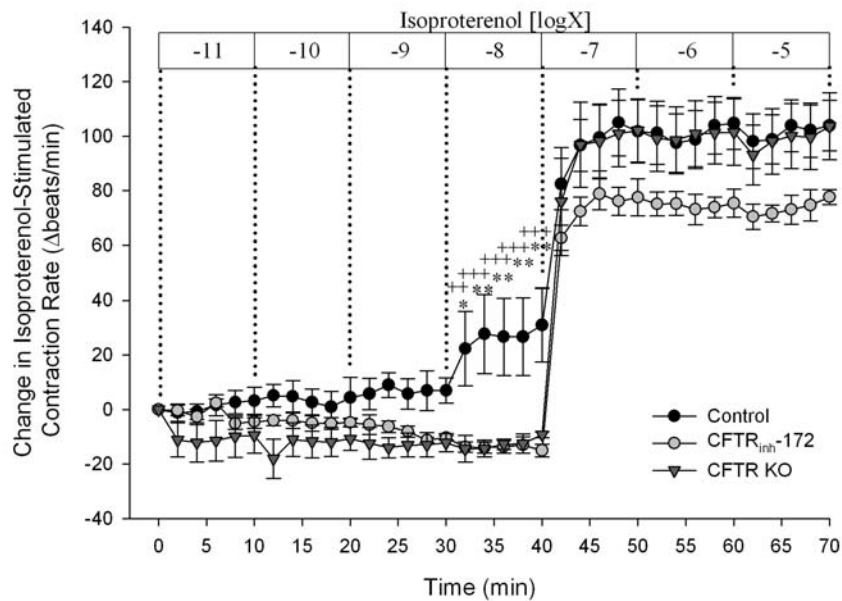
Figure 4.1. Modulation of CFTR activity preferentially alters submaximal β-adrenergic stimulated contraction rate. A. WT (n = 7), WT pretreated with $CFTR_{inh}$-172 (20 μM, 30 minutes, n = 4), or CFTR KO (n = 6) neonatal ventricular myocytes were treated with increasing doses of isoproterenol (10^{-11} M to 10^{-5} M) for 10 minutes each following an initial 10-minute baseline. Values represent responses subtracted from average baseline values. *, $P < 0.05$; **, $P < 0.01$ *vs.* $CFTR_{inh}$-172 by two-way ANOVA. ++, $P < 0.01$; +++, $P < 0.001$ *vs.* CFTR KO by two-way ANOVA. **B.** Responses from 4.1.A. were normalized and plotted. **C.** In separate experiments, CFTR KO (n = 4) or WT neonatal cardiomyocytes were pretreated with DMSO (control, n = 6), $CFTR_{inh}$-172 (20 μM, n = 5), genistein (50 μM, n = 6), or PG-01 (5 μM, n = 4) for 20 minutes prior to stimulation with submaximal (10^{-8} M) or maximal (10^{-5} M) doses of isoproterenol for 20 minutes each. Values represent maximum responses subtracted from average baseline values.

Figure 4.1 continued…

B
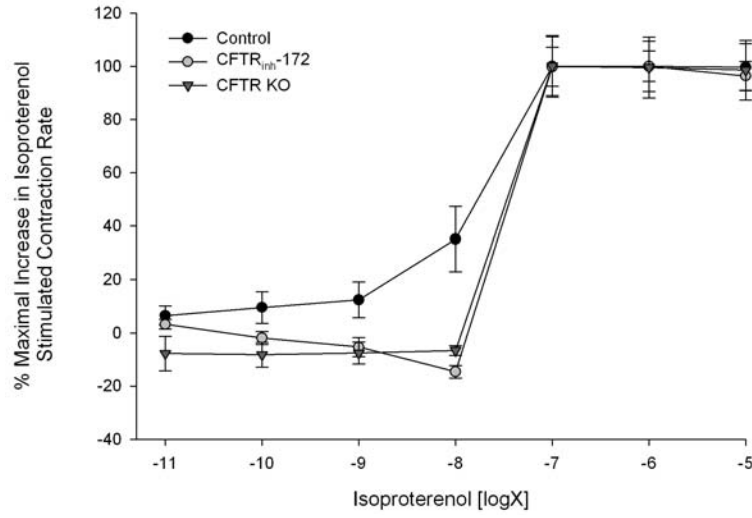
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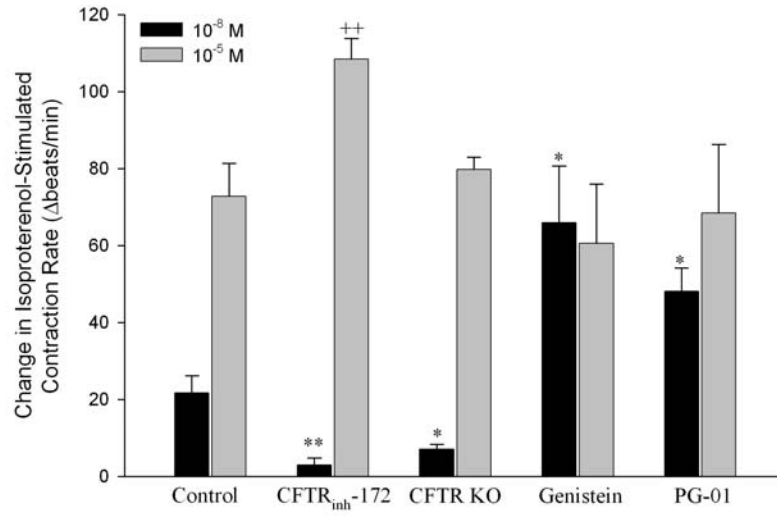
	EC$_{50}$ (nM)
Control	13.8
CFTR$_{inh}$-172	33.3
CFTR KO	38.5
Genistein	1.2
PG-01	1.5

Table 4.1. CFTR modulation alters the dose-dependency of isoproterenol-stimulated contraction rate. Similar to Figure 4.1., neonatal cardiomyocytes were subjected to increasing doses of isoproterenol from 10^{-11} M to 10^{-5} M (n ≥ 4) and responses were fitted to a standard four parameter dose response curve with calculation of half-maximal responses using SigmaPlot.

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B
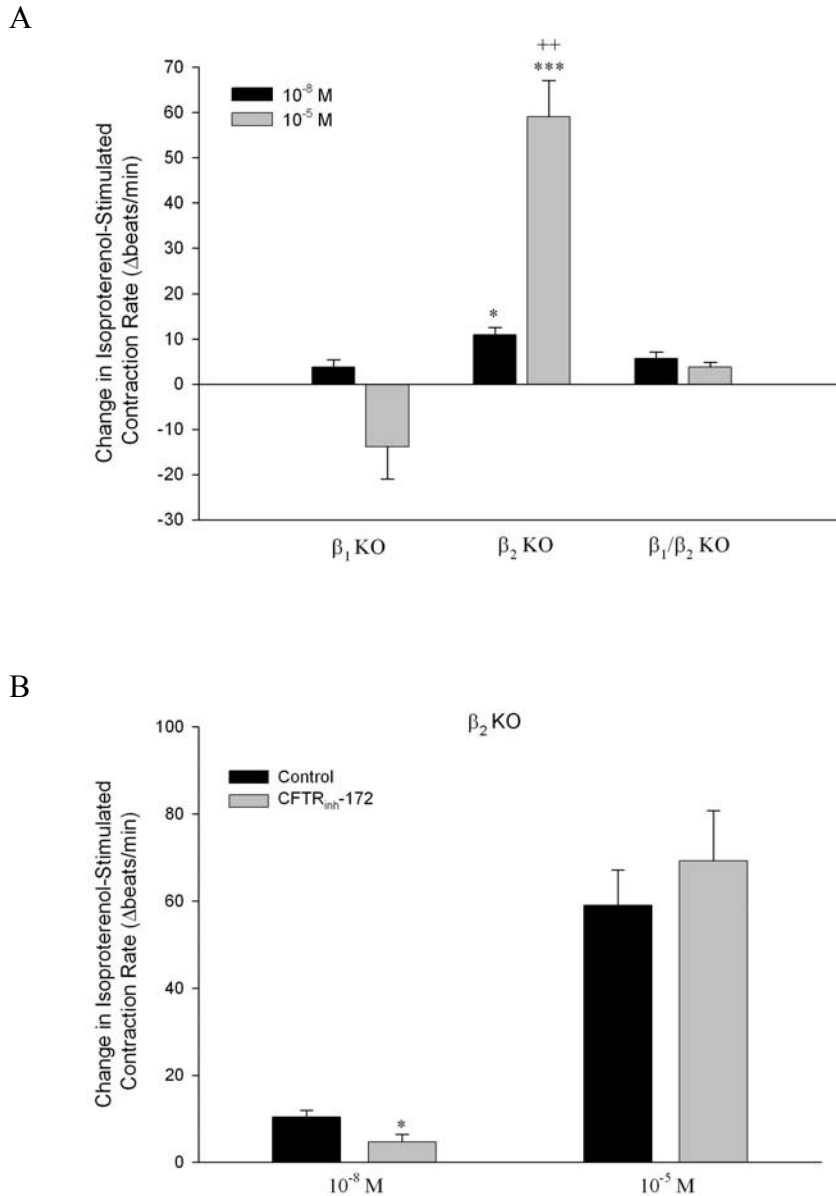
Figure 4.2. CFTR-dependent inhibition of β-adrenergic stimulated contraction rate is β₂-receptor independent. A. Neonatal ventricular myocytes from β_1 (n = 6), β_2 (n = 6), and β_1/β_2 KO (n = 6) mice were stimulated with submaximal (10^{-8} M) and maximal (10^{-5} M) doses of isoproterenol for 20 minutes each following a 10-minute baseline. Peak responses were subtracted from averaged baseline values. *, $P < 0.05$; ***, $P < 0.001$ *vs.* β_1 and $\beta_{1/2}$ KO by one-way ANOVA. ++, $P < 0.01$ *vs.* β_2 KO 10^{-8} M. **B.** In separate experiments, cardiomyocytes from β_2 KO mice were pretreated with DMSO (control, n = 6) or CFTR$_{inh}$-172 (20 μM, n = 6) for 30 minutes prior to stimulation with submaximal (10_{-8} M) and maximal (10^{-5} M) doses of isoproterenol for 20 minutes each. *, $P < 0.05$ *vs.* control by student's t-test.
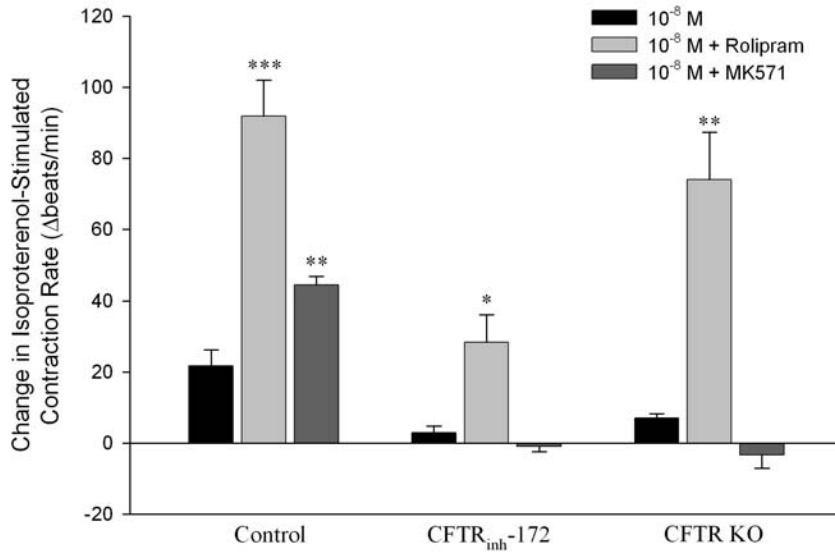
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Figure 4.3. Lack of response to submaximal β-adrenergic stimulation from loss of CFTR activity can be overcome by PDE4, but not MRP4, inhibition. Neonatal cardiomyocytes from WT mice with and without $CFTR_{inh}$-172 pretreatment (20 μM for 30 minutes) or CFTR KO mice were treated with rolipram (10 μM, n ≥ 6) or MK571 (40 μM, n ≥ 6) for 20 minutes prior to submaximal isoproterenol stimulation (10^{-8} M) for 20 minutes. *, P <0.05; **, P <0.01; ***, P < 0.001 *vs*. 10^{-8} M alone by student's t-test.
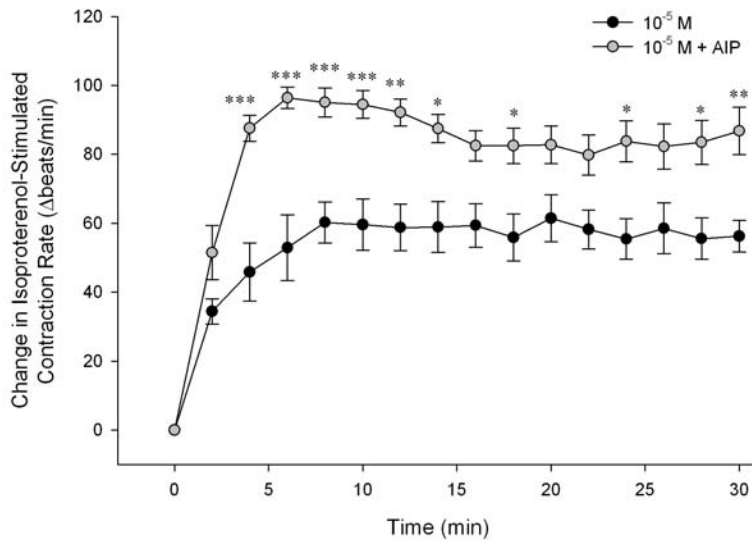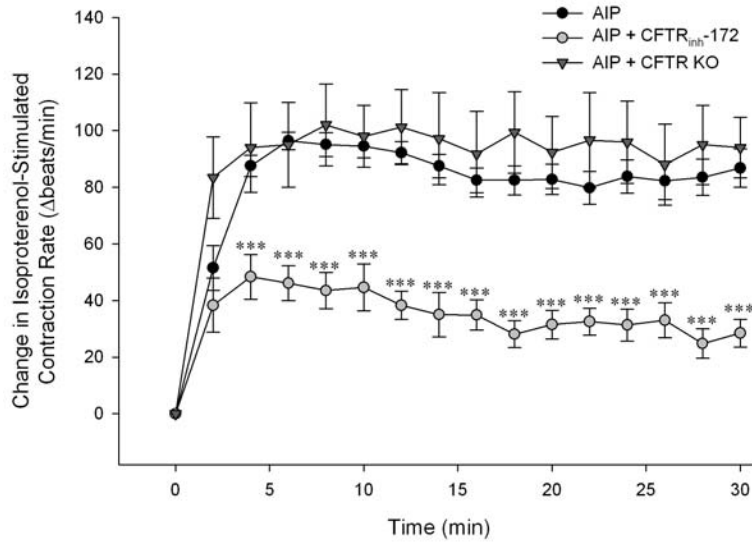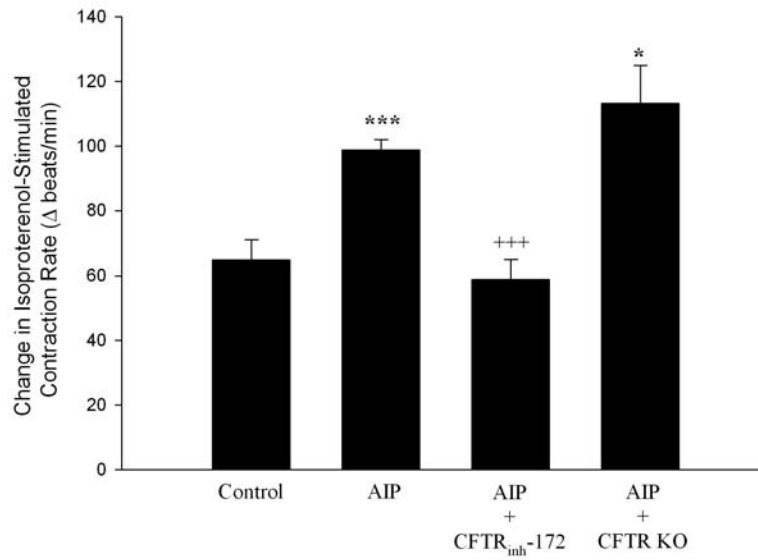
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Figure 4.4. Effect of CaMKII inhibition on maximal β-adrenergic stimulated contraction rate during pharmacoligic inhibition or genetic deletion of CFTR. Neonatal ventricular myocytes were treated with AIP (1 μM) for 30 minutes. Subsequently, cardiomyocytes from WT mice (n = 6), WT myocytes pretreated with $CFTR_{inh}$-172 (20 μM for 20 minutes, n = 8), or myocytes from CFTR KO mice (n = 6) were stimulated with 10^{-5} M isoproterenol for 30 minutes. **A and B.** Each isoproterenol-stimulated time point was subtracted from baseline values preceding stimulation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* 10^{-5} M isoproterenol alone (A). ***, $P < 0.001$ *vs.* 10^{-5} M isoproterenol plus AIP (B). **C.** Peak increase in isoproterenol-stimulated contraction rate was subtracted from baseline values preceding stimulation. *, $P < 0.05$; ***, $P < 0.001$ *vs.* control (10^{-5} M isoproterenol). +++, $P < 0.001$ *vs.* AIP (plus 10^{-5} M isoproterenol).

Figure 4.4 continued…
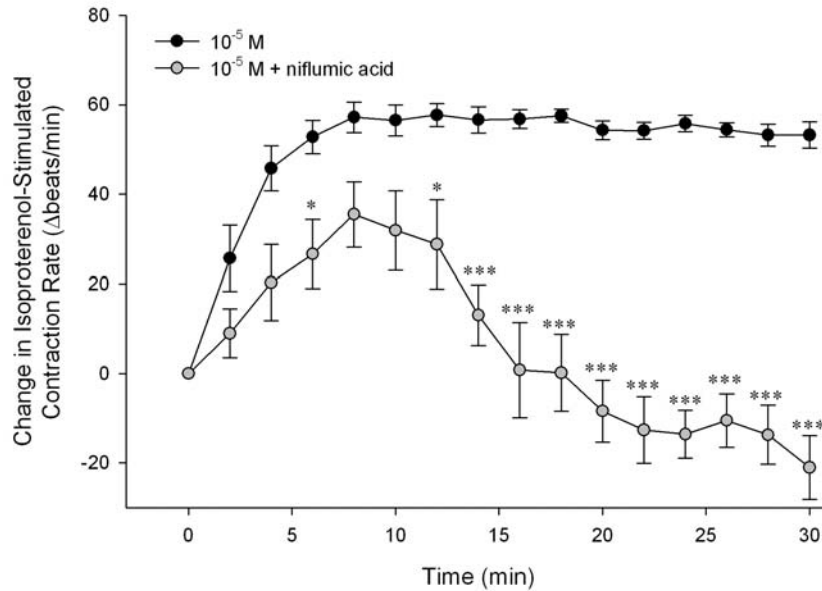
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Figure 4.5. **Effect of Ca^{2+}-activated Cl^- channel inhibition on maximal β-adrenergic stimulated contraction rate during pharmacoligic inhibition or genetic deletion of CFTR.** Neonatal ventricular myocytes were treated with niflumic acid (100 μM) for 30 minutes. Subsequently, cardiomyocytes from WT mice (n = 7), WT myocytes pretreated with $CFTR_{inh}$-172 (20 μM for 20 minutes, n = 7), or myocytes from CFTR KO mice (n = 8) were stimulated with 10^{-5} M isoproterenol for 30 minutes. **A and B.** Each isoproterenol-stimulated time point was subtracted from baseline values preceding stimulation. *, P < 0.05; ***, P < 0.001 *vs*. 10^{-5} M alone (A). **, P < 0.01 *vs*. 10^{-5} M plus niflumic acid (B). **C.** Peak increase in isoproterenol-stimulated contraction rate was subtracted from baseline values preceding stimulation. *, P < 0.05; **, P < 0.01; ***, P < 0.001 *vs*. control (10^{-5} M isoproterenol). ++, P < 0.01; +++, P < 0.001 *vs*. niflumic acid (plus 10^{-5} M isoproterenol).
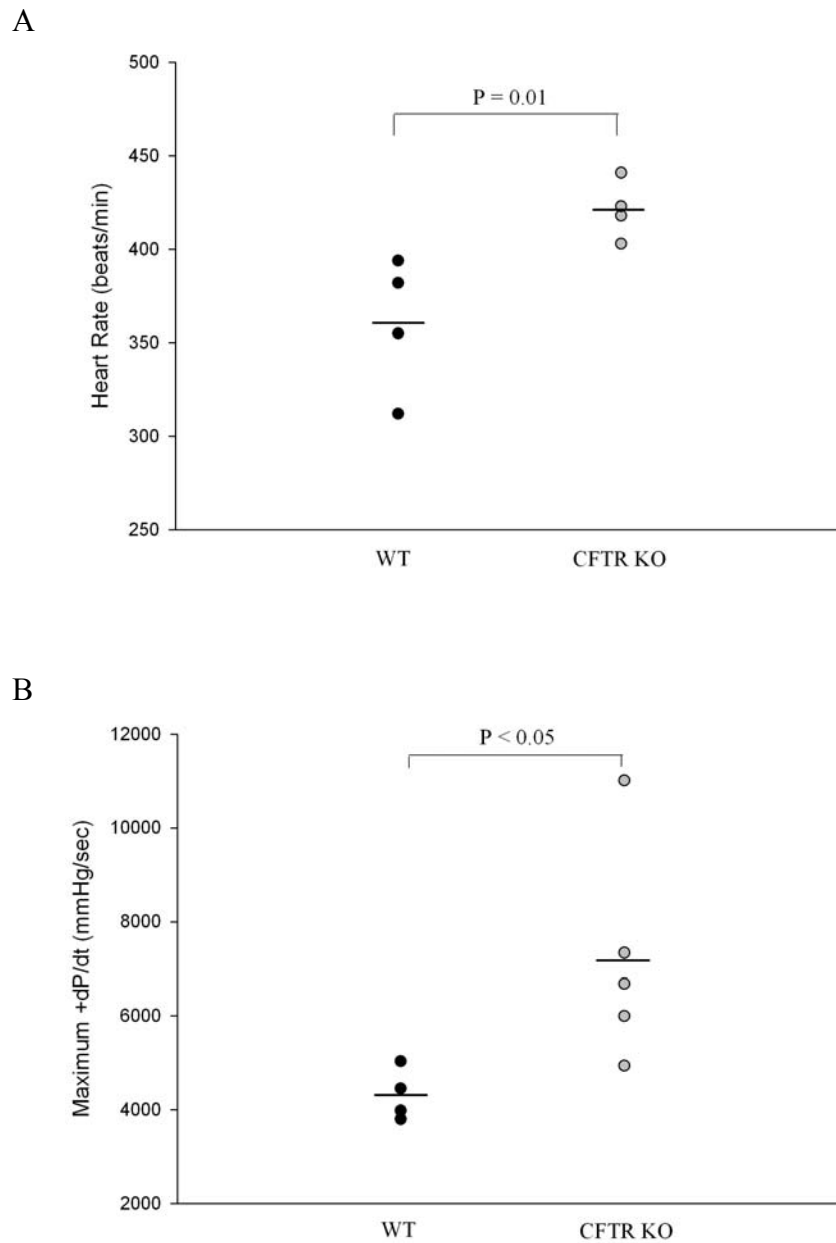
Figure 4.5 continued…

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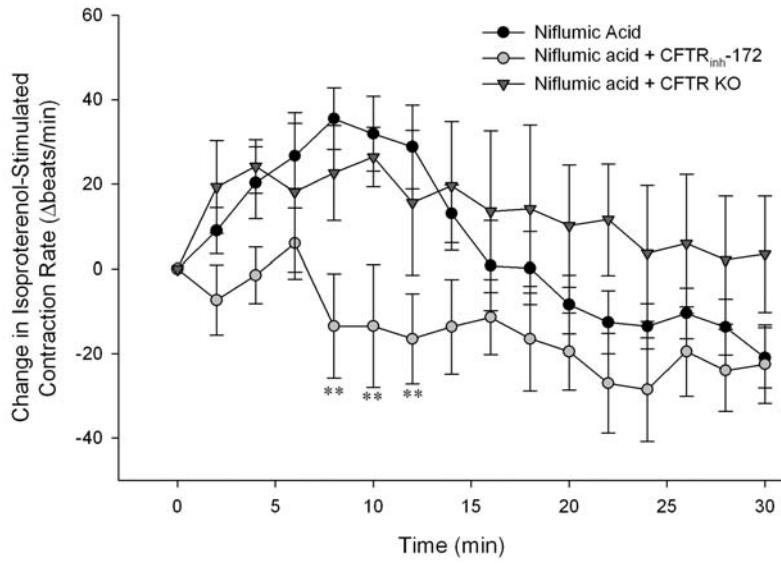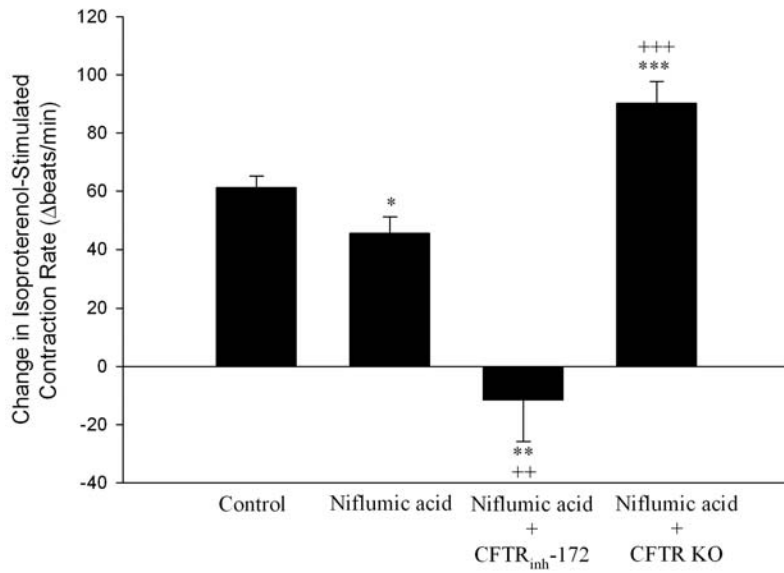
Figure 4.6. *In vivo* **heart function of CFTR KO mice at rest.** Prior to dobutamine stimulation, measurements of heart rate (A), maximal systolic pressure (+dP/dt, B), minimum diastolic pressure (-dP/dt, C), and left ventricular (LV) end-diastolic pressure (D) were obtained from WT (n = 4) and CFTR KO (n = 5) mice. Responses between WT and CFTR KO mice were compared by student's t-test. Circles represent data from individual mice and bars represent means.

Figure 4.6 continued…

C

D
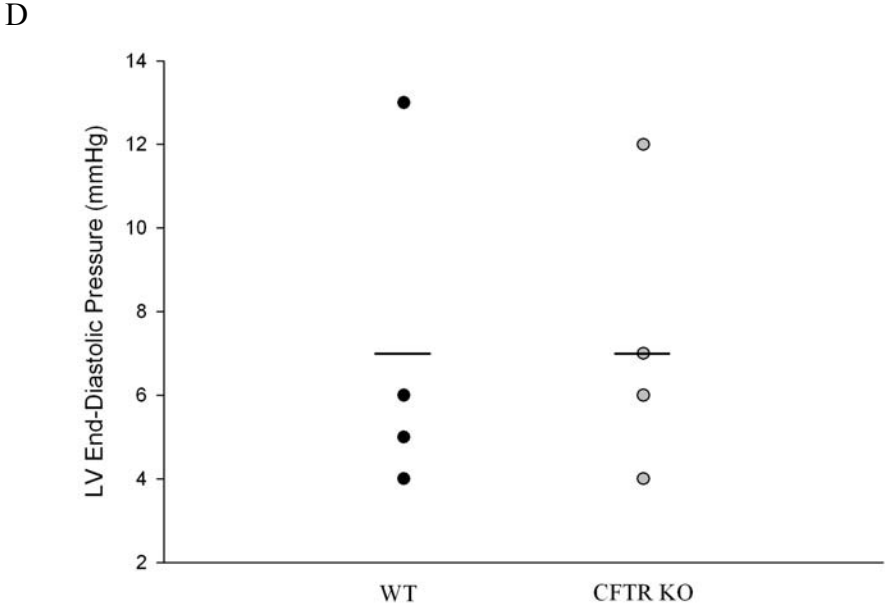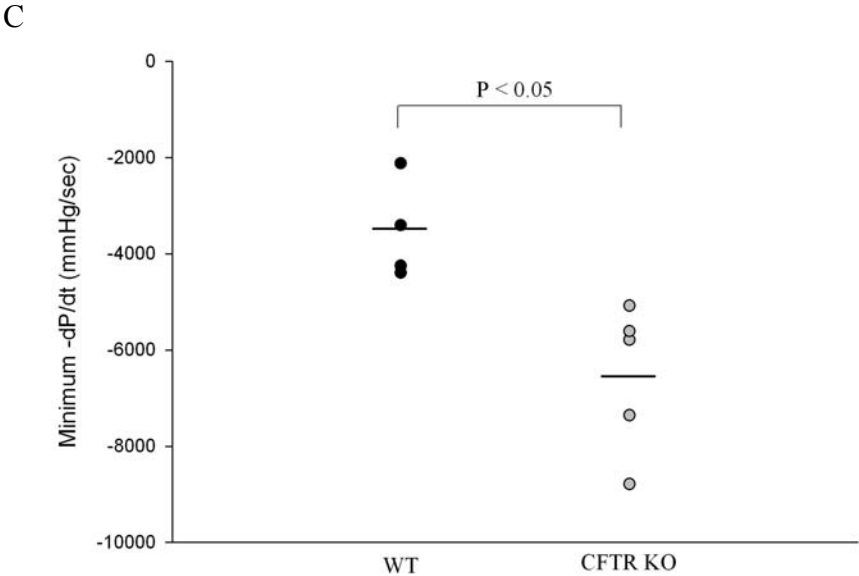
A

B
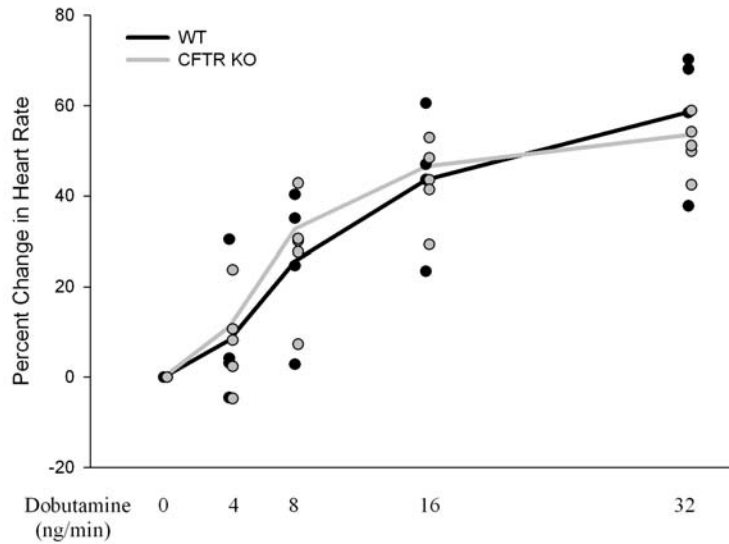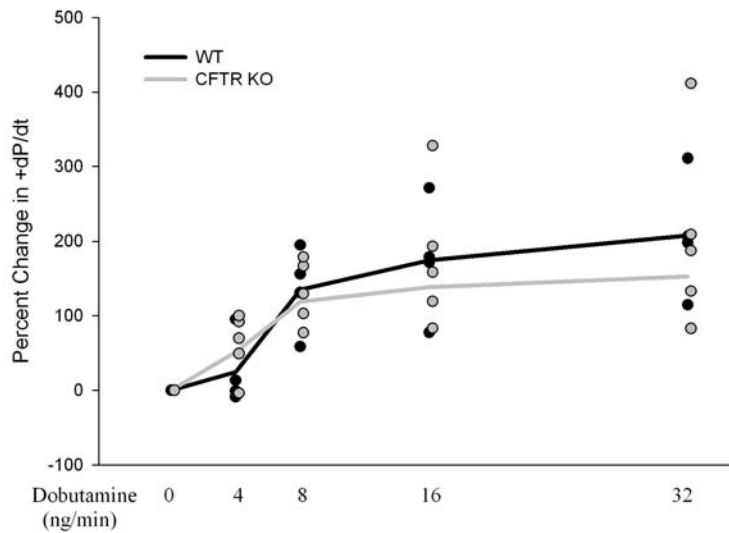
Figure 4.7. *In vivo* β-adrenergic stimulation reveals abnormalities in left-ventricular end-diastolic pressure in CFTR KO mice. Increasing doses of dobutamine were administered and subsequent responses were compared to baseline values and expressed as percent change from baseline. Circles represent data from individual mice and lines represent means. Responses between WT (n = 4) and CFTR KO (n = 5) mice at each concentration were compared by student's t-test.

Figure 4.7 continued…

C

D
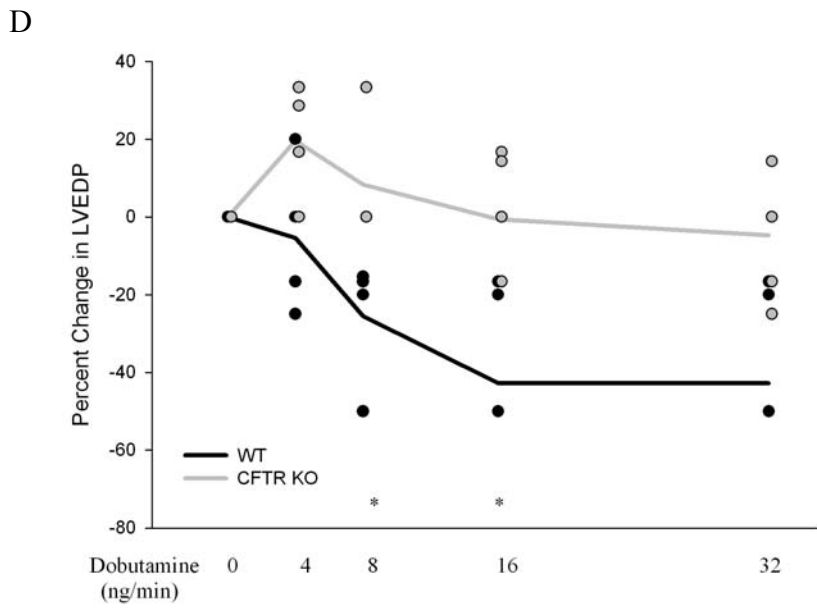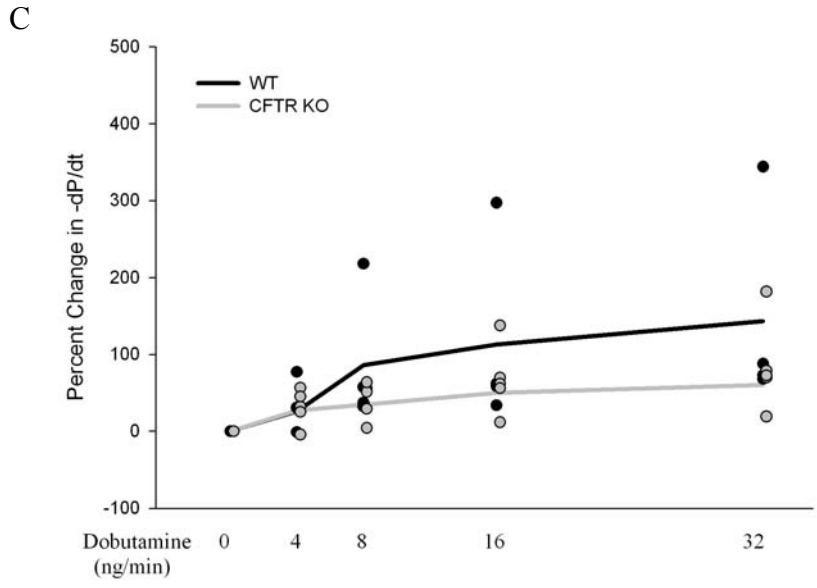
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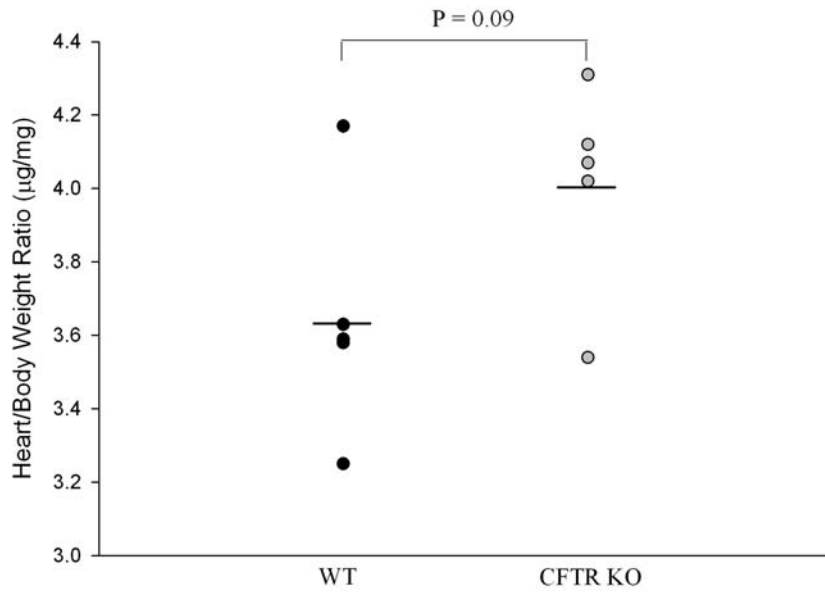
Figure 4.8. Cardiac structural disturbances in CFTR KO mice. A. Following functional studies hearts were weighed (μg) and compared to body weights (mg). **B.** In separate experiments, hearts were fixed, sectioned, and H&E stained. Areas of left ventricle, right ventricle, and interventricular septum were calculated (arbitrary units, a.u.) and normalized to body weight (mg). **C.** Representative images from WT and CFTR KO hearts.

Figure 4.8 continued…

B

C

WT   CFTR KO

WT

Male (3)

Female (2)

CFTR KO

Male (3)
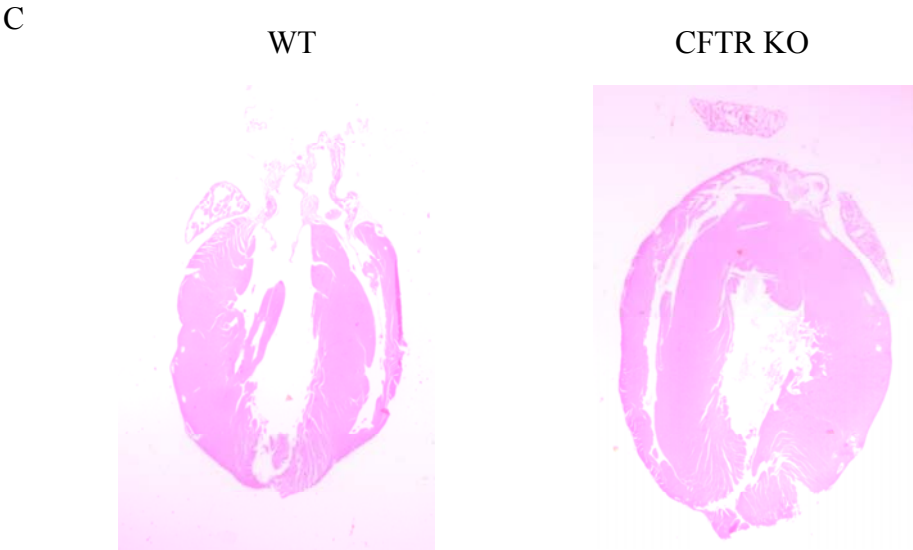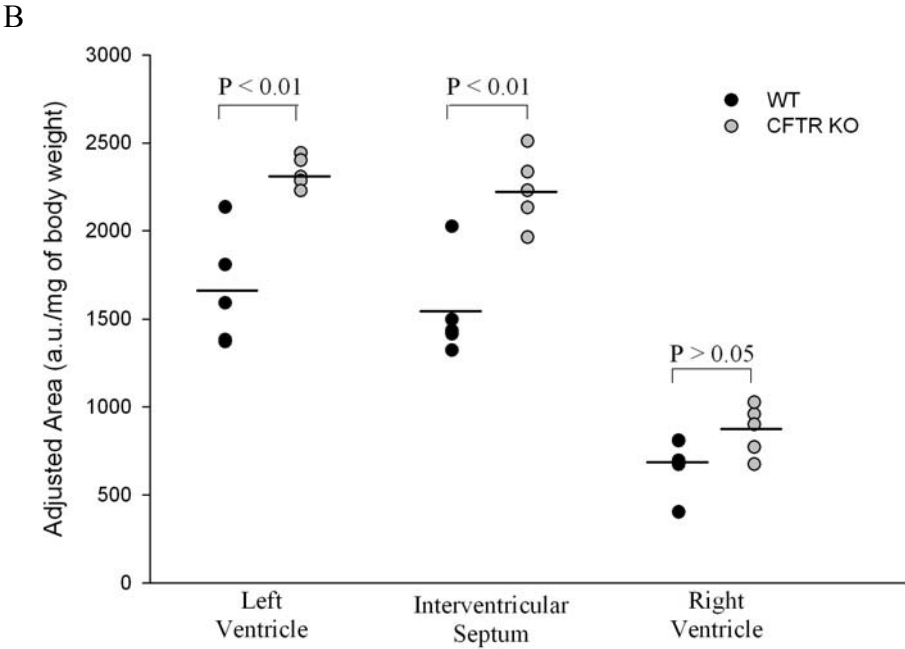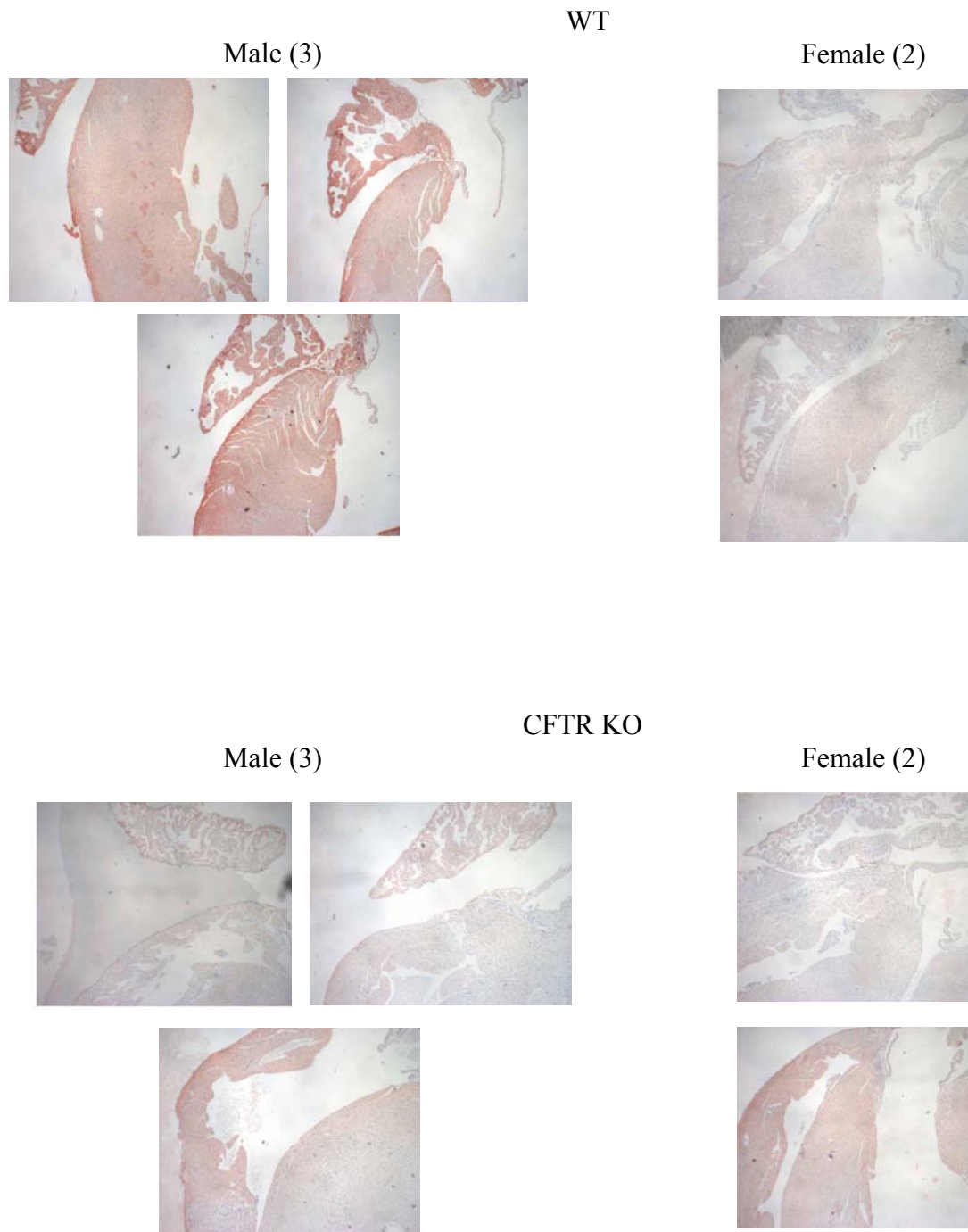
Female (2)

Figure 4.9. Genetic loss of CFTR leads to decreased expression of CaMKII in male, but not female, CFTR KO mice. Hearts from WT and CFTR KO mice were fixed, sectioned, and probed for CaMKII expression using a rabbit polyclonal anti-CaMKII antibody. Each image represents a different heart with the number of animals examined for each gender indicated in parenthesis.

WT	LV Area (a.u./mg)	IVS Area (a.u./mg)	RV Area (a.u./mg)	n	Heart/Body (µg/mg)	n
All	1657 ± 144	1538 ± 125	678 ± 74	5	3.64 ± 0.15	5
Male	1448 ± 72	1417 ± 51	628 ± 119	3	3.64 ± 0.19	4
Female	1972 [1808-2135]	1720 [1415-2025]	754 [697-811]	2	3.64	1
CFTR KO						
All	2333 ± 40 (0.002)	2234 ± 92 (0.002)	866 ± 64 (0.09)	5	4.01 ± 0.13 (0.09)	5
Male	**2383 ± 40 (0.0003)**	**2233 ± 59 (0.0005)**	844 ± 87 (0.21)	3	**4.13 ± 0.07 (0.05)**	4
Female	2257 [2228-2286] (0.23)	2236 [1963-2509] (0.33)	899 [772-1025] (0.41)	2	3.54 (n/a)	1

Table 4.2. Cardiac structural abnormalities of CFTR KO mice preferentially occur in male mice. Post-mortem structural analysis of WT and CFTR KO mouse hearts were separated according to gender and analyzed similar to Figure 4.8. Numbers in brackets indicate range of values for n < 3. Numbers in parenthesis indicate P values of CFTR KO *vs.* WT by student's t-test.

APPENDIX:

ADDITIONAL CHARACTERIZATION OF CARDIOMYOCYTE CONTRACTION RATE AND THE ION CHANNELS/TRANSPORTERS INVOLVED IN ITS REGULATION

EFFECT OF NON-SELECTIVE CFTR INHIBITORS ON CARDIOMYOCYTE CONTRACTION RATE

Prior to the availability of the selective CFTR inhibitor, $CFTR_{inh}$-172, we examined the effect of commonly used non-selective inhibitors of CFTR on cardiomyocyte contraction rate. In experiments similar to those presented in Chapter 2, syncytial neonatal ventricular myocytes were exposed to glibenclamide (100 μM, n = 7) or diphenylamine-2-carboxylate (DPC, 500 μM, n = 7). Both glibenclamide and DPC caused a rapid, significant decrease in cardiomyocyte contraction rate that recovered to near-baseline levels (Figure A.1). Beyond their wide use as inhibitors of CFTR, the consistency of responses between glibenclamide and DPC gave us confidence that our observed responses were in fact due to inhibition of CFTR and not alternate effects as their respective side targets differ from one another (glibenclamide, ATP-dependent K^+ (K_{ATP}) channels; DPC, other Cl^- channels). However, with the importance of K_{ATP} channels in the heart (1), we examined the effect of 5-hydroxydecanoic acid (5-HD) on contraction rate. Inhibition of K_{ATP} channels by 5-HD (100 μM) led to no significant alterations in baseline contraction rate compared to control (H_2O) (18.9 ± 2.7 *vs*. 11.3 ± 3.2, n = 5 each, P > 0.05 by student's t-test), thereby indicating that the effects observed with glibenclamide inhibition were due to inhibition of CFTR, not K_{ATP} channels. Although we discontinued the use of

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glibenclamide and DPC upon availability of $CFTR_{inh}$-172, these data provide further evidence

for the role of CFTR in contraction rate regulation.

ROLE OF HYPERPOLARIZATION-ACTIVATED 'FUNNY' CHANNELS IN CARDIOMYOCYTE CONTRACTION RATE

Upon determining that CFTR plays a modulatory role in neonatal ventricular myocyte

contraction rate, we wanted to understand what is responsible for driving the spontaneous

beating of cultured neonatal cardiomyocytes. Similar to sinoatrial nodal cells, 'funny' channels

(I_f) have been shown to be responsible for spontaneous beating of ventricular myocytes from rats

(2), therefore we wanted to determine if I_f is also important in neonatal cardiomyocytes from

mice. To determine this, we used the selective I_f inhibitor, ZD7288, at various doses (1-100 μM)

and monitored their effects on contraction rate. As shown in Figure A.2, inhibition of I_f was

highly dose-dependent, with full inhibition of contraction rate at higher concentrations.

Therefore, it appears that similar to rat cardiomyocytes, I_f is an integral player in driving the

spontaneous beating of neonatal mouse ventricular myocytes. This information, together with

our findings in Chapter 2, provides further evidence for the characterization of spontaneous

beating cultured neonatal cardiomyocytes.

ROLE OF HCO_3^- AND EXTERNAL ATP IN CARDIOMYOCYTE CONTRACTION RATE

While widely regarded as Cl^- channels, the ability (and importance) of HCO_3^- to permeate

CFTR is well documented and accepted. Therefore, following our observations that CFTR is

important in contraction rate, we sought to determine the effects of HCO_3^- removal on

cardiomyocyte contraction rate. To determine the effect of extracellular HCO_3^- removal on baseline and isoproterenol-stimulated contraction rate, experiments were conducted in a similar manner as those previously, except in HCO_3^-/ CO_2-free media. As seen in Figure A.3, removal of extracellular HCO_3^- did not have any inhibitory effect on baseline or isoproterenol-stimulated contraction rate. Furthermore, to eliminate any intracellular sources of HCO_3^-, we repeated the above experiments in the presence of acetazolamide (10^{-4} mol/L in HCO_3^-/CO_2-free media), a broad-spectrum inhibitor of carbonic anhydrase. Removal of both extracellular and intracellular HCO_3^- resulted in no alteration of baseline or isoproterenol-stimulated contraction rate compared to control media (Figure A.3), thereby indicating that HCO_3^- transport is not necessary for contraction rate. These data suggest that the deleterious effects of CFTR inhibition are likely the result of Cl^-, not HCO_3^-, transport.

In addition to Cl^- and HCO_3^-, CFTR has also been shown to conduct ATP (3), although its physiological significance has been questioned. To determine if the effects of CFTR inhibition on cardiomyocyte contraction rate were due, at least in part, to permeation of ATP through CFTR, we examined the effect of apyrase (10 U/mL), which hydrolyses ATP to ADP, addition to the extracellular media on cardiomyocyte contraction rate. Apyrase had no inhibitory effect on baseline contraction rate and did not prevent isoproteronol from significantly increasing cardiomyocyte contraction rate; however, this stimulated response was significantly less than control experiments (39 ± 3 *vs.* 62 ± 4 Δbeats/min, $p < 0.01$) (Figure A.4). In addition to ATP, ADP, and AMP are also important extracellular signaling molecules. Therefore, with these observations alone, we cannot rule out a role for extracellular ATP signaling in regulating cardiomyocyte contraction rate, nor the potential for CFTR to conduct ATP in cardiac myocytes.

However, we can conclude that our observed effects on cardiomyocyte contraction rate by CFTR inhibition are not primarily attributed to ATP conduction.

EFFECT OF SWELLING-ACTIVATED Cl⁻ CHANNEL INHIBITION ON CARDIOMYOCTE CONTRACTION RATE

In understanding the role of CFTR as a Cl^- channel in the heart we sought to determine if all outwardly conducting Cl^- channels exhibit the same influence on cardiomyocyte contraction rate. In addition to CFTR and Ca^{2+}-activated Cl^- channels, which we have shown to be involved in mediating contraction rate (Chapter 2), swelling-activated Cl^- channels (otherwise known as stretch- or volume-activated Cl^- channels) are also expressed in the heart. Therefore, using tamoxifen, a specific inhibitor of swelling-activated Cl^- channels at micromolar concentrations, we investigated the role of swelling-activated Cl^- channels in contraction rate. In contrast to CFTR and Ca^{2+}-activated Cl^- channels, inhibition of swelling-activated Cl^- channels led to a time-dependent decrease in baseline contraction rate, which could not be overcome by addition of isoproterenol (Figure A.5). These data show that: 1) swelling-activated Cl^- channels are vital to maintaining proper contraction rate, and 2) not all outward conducting Cl^- channels have the same role in regulating contraction rate.

EFFECT OF 4,4,-DIISOTHIOCYANOSTILBENE SULFONIC ACID (DIDS) ON CARDIOMYOCYTE CONTRACTION RATE

In numerous tissues evidence has shown that when CFTR and Cl^-/HCO_3^- anion exchangers are expressed, they functionally interact to modulate HCO_3^- secretion and pH_i. To determine whether this phenomenon occurs in ventricular myocytes and can affect contraction

rate, we examined whether the anion exchanger inhibitor DIDS (1 mM) affects cardiomyocyte contraction rate in the presence and absence of CFTR inhibition. We found that application of DIDS alone minimally affected basal contraction rate and fully prevented isoproteronol-stimulated increases in contraction rate (Figure A.6A). The initial decrease in beating upon application of DIDS was due to biological actions of DIDS on cardiomyocytes, as addition of vehicle had no inhibitory effect on either baseline or isoproteronol-stimulated contraction rate. Inhibition of CFTR by CFTR$_{inh}$-172 (20 µM) following DIDS resulted in a further decrease in contraction rate that remained unchanged upon isoproteronol stimulation (Figure A.6B). When we examined the effect of DIDS in CFTR KO, we found that DIDS minimally inhibited baseline contraction rate, but fully prevented any increase in isoproteronol-stimulated contraction rate, similar to CFTR$_{inh}$-172 (Figure A.6C). From these studies we have found that while DIDS has a minimal effect of baseline contraction rate, however, it completely blocks isoproteronol-stimulated contraction rate, regardless of CFTR activity.

One caveat to be considered when interpreting these studies is that DIDS is a non-selective inhibitor of anion exchange. In addition to anion exchangers, DIDS has also been used to inhibit the Na:HCO$_3^-$ contransporter (NBC), swelling-activated and Ca^{2+}-activated Cl$^-$ channels. As the inhibition of contraction rate produced by DIDS did not resemble that of tamoxifen (Figure A.5) or niflumic acid (Chapter 2, 4), which selectively inhibit swelling- or Ca^{2+}-activated Cl$^-$ channels, respectively, we examined if the observed effect with DIDS could be due to inhibition of the NBC. To determine this we performed similar experiments with the selective NBC inhibitor, SO859. SO859 (10 µM) had no inhibitory effect on baseline or isoproteronol-stimulated increases in cardiomyoctye contraction rate (data not shown, n = 4, P > 0.05), thereby indicating that inhibition of NBC by DIDS does not explain the effect of DIDS on

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baseline or isoproteronol-stimulated contraction rate. As a result, we conclude that the observed

inhibitory effects of DIDS are due to inhibition of anion exchange. As we have previously

shown that HCO_3^- removal has no inhibitory effect on contraction rate (Figure A.3), we believe

that this involves Cl^-/OH^-, not Cl^-/HCO_3^-, exchange.

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3. Prat AG, Reisin IL, Ausiello DA, Cantiello HF. Cellular ATP release by the cystic fibrosis transmembrane conductance regulator. Am J Physiol. 1996 Feb;270(2 Pt 1):C538-45.

FIGURES

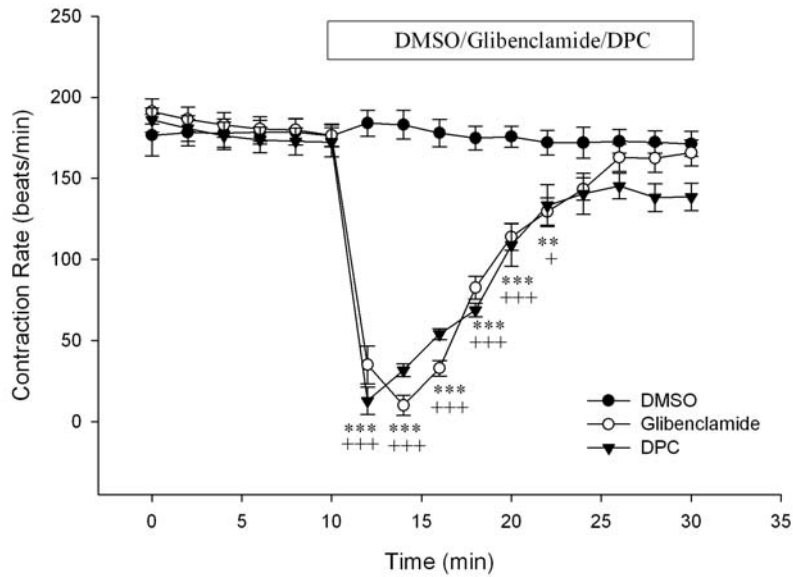**Figure A.1. Effect of glibenclamide and DPC on cardiomyocyte contraction rate.** Similar to experiments in Chapter 2, neonatal cardiomyocytes were treated with DMSO (n = 7), glibenclamide (100 μM, n = 7), or DPC (500 μM, n = 7) for 20 minutes following a 10-minute baseline. **, $P < 0.01$; ***, $P < 0.001$ glibenclamide *vs*. DMSO by two-way ANOVA. +, $P < 0.05$; +++, $P < 0.001$ DPC *vs*. DMSO by two-way ANOVA.

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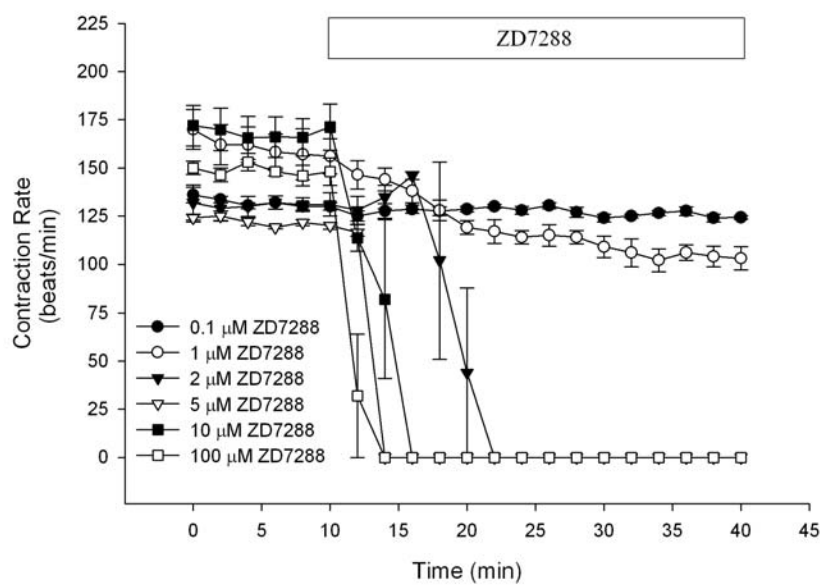
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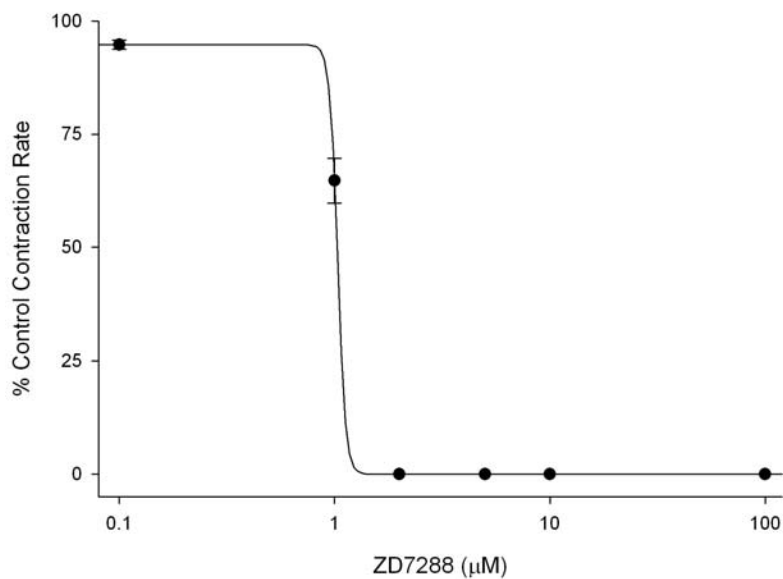
Figure A.2. Role of hyperpolarization-activate 'funny' channels in cardiomyocyte contraction rate. Syncytial WT ventricular myocytes were treated with ZD7288 (0.1-100 μM, n = 3) for 30 minutes, following a 10-minute baseline. Data are expressed as actual contraction rates (A) or peak inhibitory compared to baseline (B).

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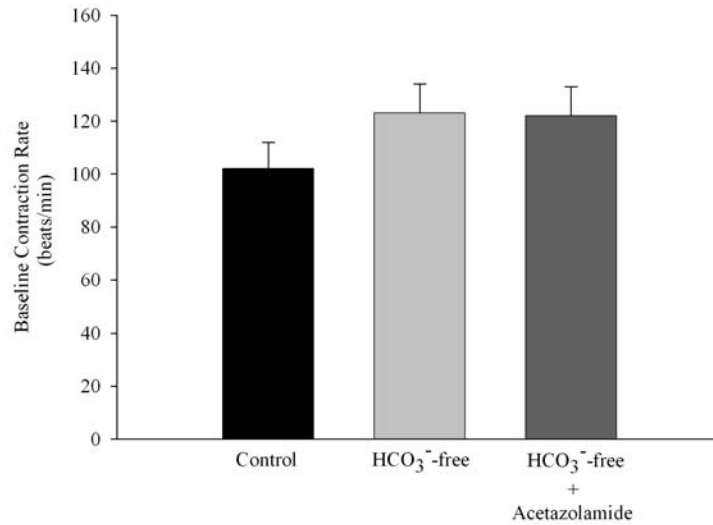
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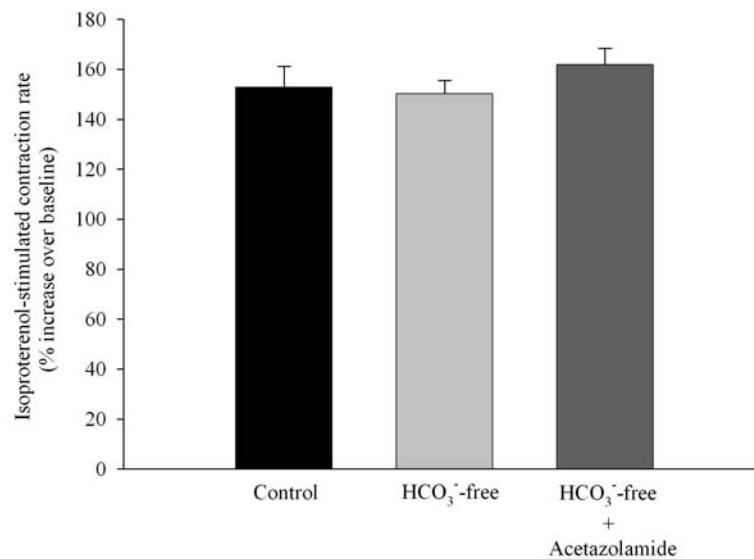
Figure A.3. Effect of HCO_3^- removal on cardiomyocyte contraction rate. Baseline (A) and isoproterenol-stimulated (B, 10 μM) contraction rates of WT syncytial cardiomyocytes were examined in the presence (control, n = 6) or absence of extracellular HCO_3^- (HCO_3^--free, n = 7), or total HCO_3^- removal (HCO_3^--free + 100 μM acetazolamide, n = 7). Baseline data are expressed as actual values, whereas isoproterenol-stimulated data are expressed as peak responses subtracted from their respective baseline values. Neither group was statistically significant (P > 0.05) from control data.
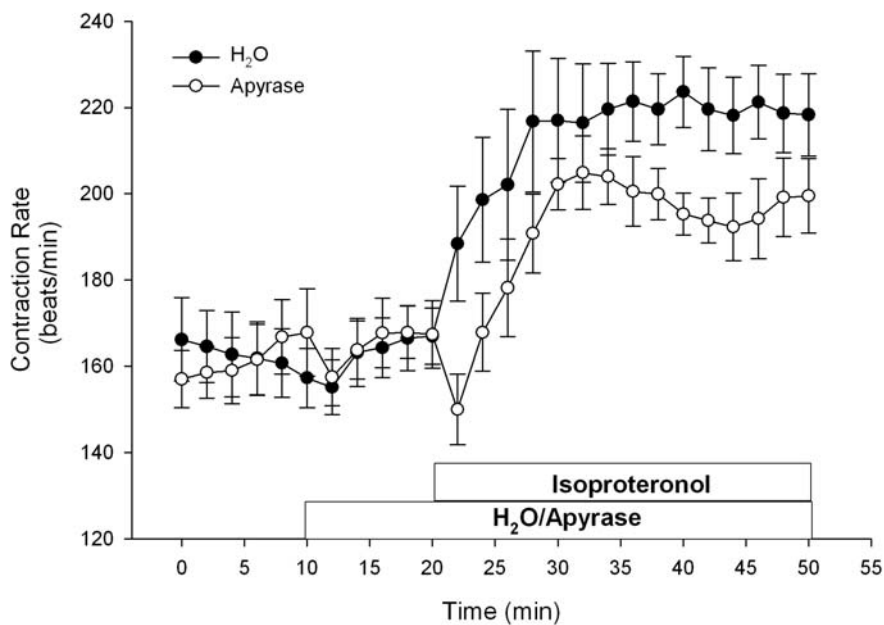
Figure A. 4. Role of external ATP in cardiomyocyte contraction rate. Syncytial WT ventricular myocytes were treated with H_2O (n = 5) or Apyrase (10 U/mL, n = 5) for 10 minutes prior to stimulation. Subsequently, myocytes were stimulated with isoproterenol (10 μM) for an additional 30 minutes.
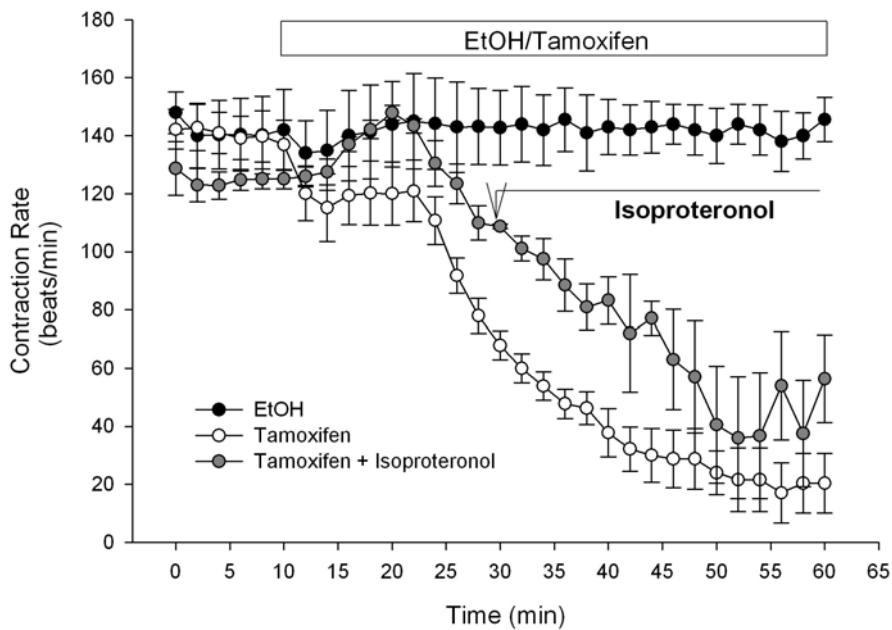
Figure A.5. Effect of swelling-activated Cl⁻ channel inhibition on cardiomyocyte contraction rate. Syncytial WT ventricular myocytes were treated with tamoxifen (10µM, n = 5) or vehicle control (n = 3) for 50 minutes following a 10-minute baseline. In additional experiments (n = 4) isoproterenol (10 µM) was added 20 minutes after tamoxifen for 30 minutes.

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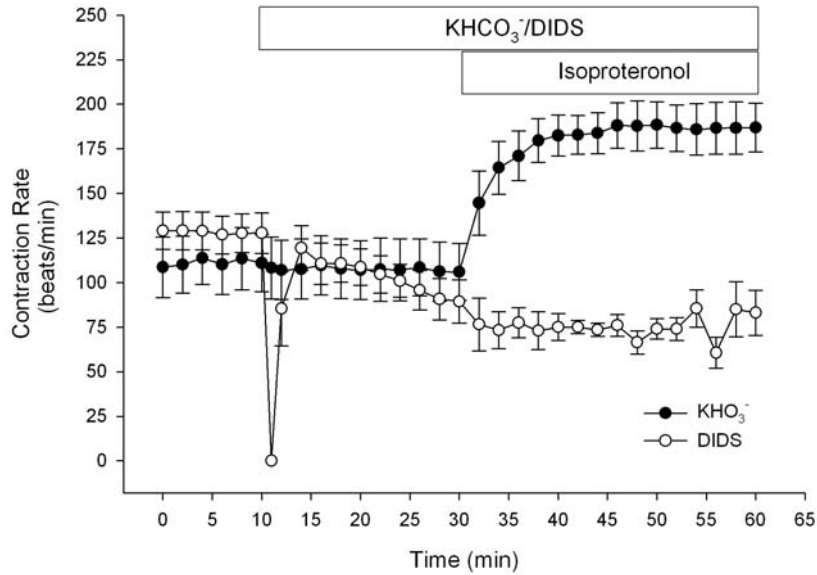
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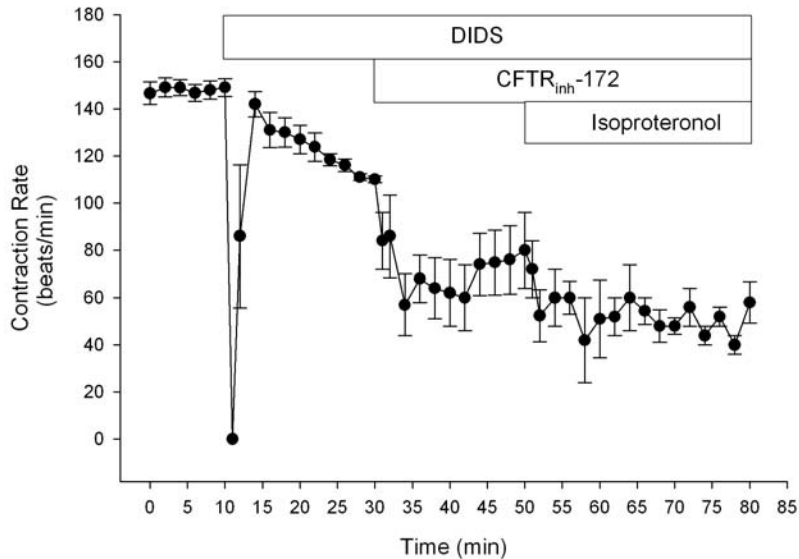
Figure A.6. Effect of DIDS on cardiomyocyte contraction rate. A and B. Syncytial WT ventricular myocytes were treated with DIDS (1 mM, n = 6), vehicle control (0.1 M KHCO$_3$, n = 5), and/or CFTR$_{inh}$-172 (20 μM, n = 5) for 20 minutes following a 10-minute baseline. **C.** Syncytial CFTR KO ventricular myocytes were treated with DIDS (1 mM, n = 6) for 20 minutes following a 10-minute baseline. Subsequently, all cell were treated with isoproteronol (10 μM) for 30 minutes.

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Figure A.6 continued…

C
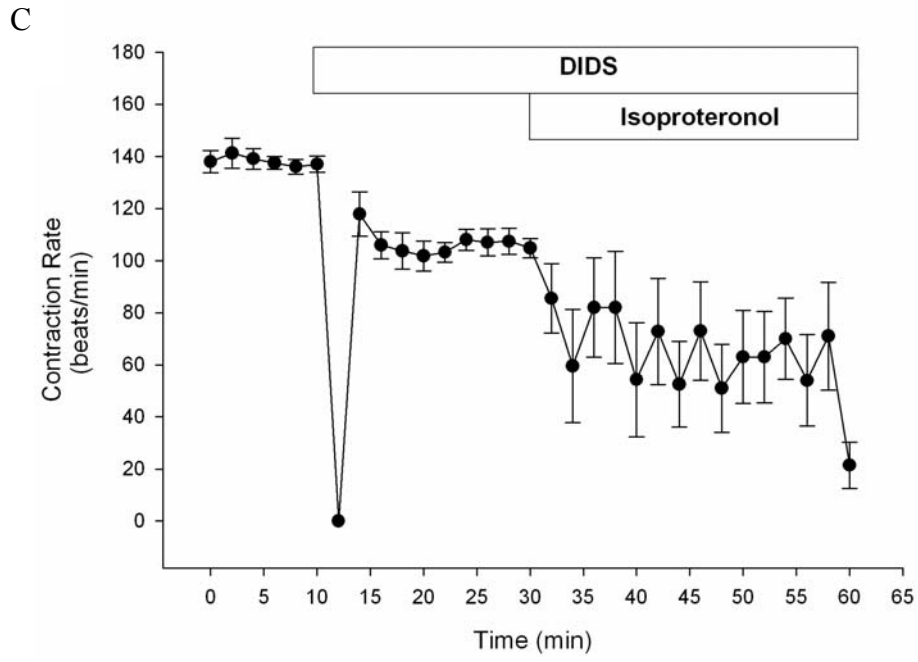
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1. **Sellers ZM**, V De Arcangelis, Y Xiang, PM Best. Disruption of CFTR in cardiomyocytes requires CaMKII and Ca^{2+}-activated Cl^- channels to maintain contraction rate. *Submitted to Journal of Physiology.*

2. **Sellers ZM**, AP Naren, Y Xiang, PM Best. Multi-drug resistant protein 4 in cardiac myocytes: another player in the regulation of intracellular cAMP and β-adrenergic signaling. *In preparation for submission to AJP-Heart and Circulatory Physiology.*

3. **Sellers ZM**, D Soto, Y Xiang, PM Best. *In vitro* and *in vivo* examination of CFTR in β-adrenergic stimulated heart function.

4. **Sellers ZM**, AJ Smith, M Soleimani, U Seidler, KE Barrett, H Dong. Central role for carbonic anhydrase and PAT-1 in STa-stimulated duodenal HCO_3^- secretion in CFTR knockout mice.

5. Dong H, **ZM Sellers**, JY Chow, AJ Smith, KE Barrett. Expression and function of TRP channel in duodenal mucosal regulation of Ca^{2+}-dependent ion transport.

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7. Solicited article for *The Physiologist* (a publication of the American Physiological Society) examining the activities of Physiology Ph.D. graduates. Scheduled for publication in Summer-Fall 2010.

ABSTRACTS PRESENTED AT INTERNATIONAL CONFERENCES

1. Crombie D, S Keely, DL Hogan, **Z Sellers**, K Barrett, J Isenberg, V Pratha. PGE_2-stimulated human duodenal epithelial bicarbonate secretion involves MAPK (ERK) activation. Gastroenterology 2001; 120 (5): A247. Digestive Disease Week.

2. Childs D, D Crombie, V Pratha, **Z Sellers**, DL Hogan, JI Isenberg. Heat stable toxin of E. Coli (STa) stimulated duodenal mucosal bicarbonate secretion in cystic fibrosis knockout mice. Gastroenterology 2001; 120 (5): A527. Digestive Disease Week.

3. Tuo B, V Pratha, **Z Sellers**, SP Rao, JI Isenberg. $CagA^+/VacA^+$ positivity of Helicobacter pylori mediates the inhibition of agonist-stimulated duodenal mucosal bicarbonate secretion (DMBS) in mice in vitro. Gastroenterology 2002;122 (4): A227. Digestive Disease Week.

4. Rao SP, **Z Sellers**, P Paulus, G Perez-Perez, M Blaser, JI Isenberg, V Pratha. H. pylori alters bicarbonate secretion and signal transduction events in human duodenal mucosa in vitro: a CagA/VacA-dependent effect. Gastroenterology 2002;122 (4): A515. Digestive Disease Week.

5. Pompa D, **Z Sellers**, P Paulus, JI Isenberg, R Mittal, V Pratha. Human esophageal epithelium in vitro: a novel technique using endoscopic biopsies. Gastroenterology 2002;122 (4): A112. Digestive Disease Week.

6. Tuo B, P Paulus, **Z Sellers**, JI Isenberg. 5-hydroxytryptamine $_4$ receptors mediate 5-hydroxytryptamine-induced duodenal mucosal bicarbonate secretion in mice. Gastroenterology 2003;124 (4): A452. Digestive Disease Week.

7. **Sellers Z**, B Tuo, S Keely, JI Isenberg. ERK-phosphorylation regulates basal, but not agonist-stimulated duodenal mucosal bicarbonate secretion in murine duodenum in vitro. Gastroenterology 2003;124 (4): A444. Digestive Disease Week.

8. **Sellers Z**, P Kumar, J Steinbach, N Khalili, B Macias, D Watenpaugh, E. Groppo, AR Hargens, V Pratha. Colonic motility is unaltered by lower body negative pressure exercise during prolonged antiorthostatic bedrest-implications for spaceflight. Gastroenterology 2003;124 (4): A572. Digestive Disease Week.

9. Dohil R, R Newbury, **Z Sellers**, R Deutsch, J Schneider. The evaluation of gastrointestinal disease and effect of Omeprazole therapy in children with cystinosis receiving cysteamine. Gastroenterology 2003;124 (4): A516. Digestive Disease Week.

10. Dong H, **ZM Sellers**, JYC Chow, KE Barrett. Intracellular Ca^{2+} handling mechanisms during duodenal mucosal ion transport in mice. Gastroenterology 2004;126 (4): A534. Digestive Disease Week.

11. **Sellers ZM**, AJ Smith, H Dong, KE Barrett. STa stimulates a novel bicarbonate secretory pathway in CFTR knockout mice. Gastroenterology 2004;126 (4): A297. Digestive Disease Week.

12. Dong H, **ZM Sellers**, A Smith, X Wang, KE Barrett. Role of Na^+/Ca^{2+} exchange in the regulation of duodenal mucosal bicarbonate secretion. Experimental Biology and XXXV International Congress of Physiological Sciences, 2005.

13. **Sellers ZM**, H Dong, KE Barrett. ST_a and uroguanylin stimulate distinct guanylyl cyclase C-independent duodenal bicarbonate secretory pathways in mice. Gastroenterology 2005;128 (4): A116. Digestive Disease Week.
14. **Sellers ZM**, A Smith, H Dong, KE Barrett. Differential roles of carbonic anhydrase and Na^+/HCO_3^- cotransporter in ST_a-stimulated duodenal mucosal bicarbonate secretion in normal and cystic fibrosis mice. Gastroenterology 2005;128 (4): A347. Digestive Disease Week.
15. Dong H, A Smith, **ZM Sellers**, JYC Chow, KE Barrett. Role of TRPV channels in the regulation of duodenal mucosal bicarbonate secretion. Gastroenterology 2005;128 (4): A365. Digestive Disease Week.
16. Dohil R, M Fidler, R Newbury, **Z Sellers**, R Deutsch, J Schneider. Esomeprazole Therapy for Gastric Acid Hypersecretion in Children Receiving Regular Cysteamine. Gastroenterology 2005;128 (4): A300. Digestive Disease Week.
17. **Sellers ZM**, Y Xiang, PM Best. CFTR is involved in the regulation of cardiomyocyte contraction rate. Pediatric Pulmonology 2007; 30: 218. North American Cystic Fibrosis Conference.
18. **Sellers ZM**, Y Xiang, PM Best. Regulation of basal and beta-adrenergic-stimulated ventricular cardiomyocyte contraction rate involves distinct populations of chloride channels. Experimental Biology 2008.
19. **Sellers ZM**, Y Xiang, PM Best. Inhibition of the cystic fibrosis transmembrane conductance regulator in cardiomyocytes causes alterations in PKA activity and intracellular Ca^{2+}. The Beijing Joint Conference of Physiological Sciences 2008.
20. **Sellers ZM**, Y Xiang, PM Best. CFTR inhibition leads to stimulation of calcium-activated Cl^- channels through alterations in intracellular Ca^{2+}. North American Cystic Fibrosis Conference 2008.
21. **Sellers ZM**, AP. Naren, Yang Xiang, PM Best. Functional Interaction of CFTR and MRP4 in mediating β_1-adrenergic receptor stimulated increases in cardiomyocyte contraction rate. Experimental Biology 2009.
22. **Sellers ZM**, Y Xiang, PM Best. CFTR is responsible for modulating local β_1-adrenergic receptor stimulated increases in cardiomyocyte contraction rate. Experimental Biology 2009.

INVITED PRESENTATIONS

2005   West Coast Salt and Water Club, 24th Annual Meeting
            "*Escherichia coli* heat-stable enterotoxin (STa) and bicarbonate secretion:
             implications for cystic fibrosis"

2005   Digestive Disease Week
            "ST_a and uroguanylin stimulate distinct guanylyl cyclase C-independent
             duodenal bicarbonate secretory pathways in mice"

2008, 2009 Experimental Biology, Undergraduate Student Orientation
            "Tips for attending and presenting a poster"

2008	Department of Physiology, Cardiovascular Group
	University of Tennessee-Memphis
	"CFTR in the heart: redundant or important protein for cardiac function"

2008	ICLCS: Institute for Chemistry Literacy Through Computational Science
	University of Illinois at Urbana-Champaign
	"Cystic fibrosis: applying lessons from the clinic and research to the classroom"

2009	Department of Molecular and Integrative Physiology, Department Seminar
	University of Illinois at Urbana-Champaign
	"Understanding the role of CFTR in the heart: a look at cardiomyocyte contraction rate"

2009	College of Medicine, Clinical Research Conference Series
	University of Illinois at Urbana-Champaign
	"Cystic fibrosis and heart disease?"

2009	Department of Molecular and Integrative Physiology, Retreat
	University of Illinois at Urbana-Champaign
	"Phenotyping CFTR knockout mice to explore the cardiac health of CF patients"

RESEARCH GRANT SUPPORT

2006-2008	Author and Co-Investigator (P.I. Hui Dong, UCSD)
	Pilot and Feasibility Grant, Cystic Fibrosis Foundation
	"Identification of a non-CFTR bicarbonate secretory pathway stimulated by STa"

2006-2007	Author and primary researcher (P.I. Philip Best, UIUC)
	Research Board Grant, University of Illinois at Urbana-Champaign
	"A small peptide modulator of cardiac calcium current"

2007	Principal Investigator
	College of Medicine, Hazel I. Craig Fellowship, University of Illinois at Urbana-Champaign
	"Exploring functional interactions between CFTR and the hyperpolarization-activated current in the heart"

2008-2010	Principal Investigator
	Pre-doctoral Fellowship, American Heart Association
	"Role of CFTR in modulation of cardiomyocyte contraction rate"

COMMITTEE SERVICE

2006, 2007	Annual Research Symposium Committee
	Department of Molecular and Integrative Physiology
	University of Illinois at Urbana-Champaign

2008-2010	Career Opportunities in Physiology Committee American Physiological Society

2007-present Founder and Coordinator
Cystic Fibrosis Focus Group
University of Illinois at Urbana-Champaign

2008-present Ad hoc consultant on cystic fibrosis
ICLCS: Institute for Chemistry Literacy Through Computational Science One
  Gene Chemistry Educational Module
University of Illinois at Urbana-Champaign

PROFESSIONAL ASSOCIATIONS

2005-2009 Sigma Xi Research Society

2006-2009 Collegium Aesculapium

2007-2008 American Gastroenterological Association

2007-2008 American Association for the Advancement of Science

2005-present American Physiological Society

2007-present American Academy of Pediatrics

2008-present American Medical Student Association

TEACHING

2004 Guest Lecturer
Visualizing the Microworld, University of California, San Diego
"Helicobacter pylori"

2006 Guest Lecturer
MCB404 Systems and Integrative Physiology, Molecular and Cellular Biology,
  University of Illinois at Urbana-Champaign
"Epithelial Transport"

2006-2007 Assistant Surgeon
Cardiovascular Pig Lab, Molecular and Cellular Biology, University of Illinois at
  Urbana-Champaign

2007	Teaching Assistant
	MCB404 Systems and Integrative Physiology, Molecular and Cellular Biology, University of Illinois at Urbana-Champaign

2007-2008   Teaching Assistant
Medical Physiology, College of Medicine, University of Illinois at Urbana-Champaign

2008-2010   Head Surgeon
Cardiovascular Pig Lab, Molecular and Cellular Biology, University of Illinois at Urbana-Champaign

COMMUNITY SERVICE RELATED TO PROFESSIONAL WORK

2002-2005   Surgical Day Coordinator
Fresh Start Surgical Gifts, San Diego, CA

2003-2005   Volunteer
Free Health Clinic, University of California, San Diego

2005   Participant
Cystic Fibrosis Great Strides Walk, Urbana, Illinois

2008   Presentations to 5-12 year olds on cystic fibrosis
Society of Women Engineers Take Your Sons/Daughters to Work Day

2009-present Hermes Free Health Clinic
UIUC College of Medicine, Urbana, IL