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## MRP4 and CFTR in the regulation of cAMP and $\beta$ -adrenergic contraction in cardiac myocytes

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### Abstract

Spatiotemporal regulation of cAMP in cardiac myocytes is integral to regulating the diverse functions downstream of  $\beta$ -adrenergic stimulation. The activities of cAMP phosphodiesterases modulate critical and well-studied cellular processes. Recently, in epithelial and smooth muscle cells, it was found that the multi-drug resistant protein 4 (MRP4) acts as a cAMP efflux pump to regulate intracellular cAMP levels and alter effector function, including activation of the cAMP-stimulated  $\text{Cl}^-$  channel, CFTR (cystic fibrosis transmembrane conductance regulator). In the current study we investigated the potential role of MRP4 in regulating intracellular cAMP and  $\beta$ -adrenergic stimulated contraction rate in cardiac myocytes. Cultured neonatal ventricular myocytes were used for all experiments. In addition to wildtype mice,  $\beta_1$ -,  $\beta_2$ -,  $\beta_1/\beta_2$ -adrenoceptor, and CFTR knockout mice were used. MRP4 expression was probed via Western blot, intracellular cAMP was measured by fluorescence resonance energy transfer, while the functional role of MRP4 was assayed via monitoring of isoproterenol-stimulated contraction rate. We found that MRP4 is expressed in mouse neonatal ventricular myocytes. A pharmacological inhibitor of MRP4, MK571, potentiated submaximal isoproterenol-stimulated cAMP accumulation and cardiomyocyte contraction rate via  $\beta_1$ -adrenoceptors. CFTR expression was critical for submaximal isoproterenol-stimulated contraction rate. Interestingly, MRP4-dependent changes in contraction rate were CFTR-dependent, however, PDE4-dependent potentiation of contraction rate was CFTR-independent. We have shown, for the first time, a role for MRP4 in the regulation of cAMP in cardiac myocytes and involvement of CFTR in  $\beta$ -adrenergic stimulated contraction. Together with phosphodiesterases, MRP4 must be considered when examining cAMP regulation in cardiac myocytes.

### Keywords

MRP4; cAMP;  $\beta$ -adrenergic signaling; cardiac myocytes; CFTR

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## 1. 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 commented on by Kass (2008). Multi-layer compartmentalization of cAMP signaling occurs as a result of differential localization of  $\beta$ -adrenoceptor subtypes, adenylyl cyclase, PKA, Epac (exchange protein activated by cAMP), and phosphodiesterases (PDEs) via anchoring proteins, such as A-kinase anchoring proteins (AKAPs) (Dodge-Kafka et al., 2006). 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  $\beta$ -adrenergic-stimulated increases in cAMP are predominantly regulated by PDE4 with PDE3 playing a minor role (Jurevicius et al., 2003; Leroy et al., 2008).

Multi-drug resistance protein 4 (MRP4) is a member of the ATP-binding cassette transporter superfamily and has been shown to transport antiviral nucleoside-based drugs (Schuetz et al., 1999) and endogenous molecules involved in cellular communication and signaling, including, but not limited to, organic anions, prostaglandins, conjugated steroids, and cyclic nucleotides (Russel et al., 2008). Of particular interest, MRP4 has been shown to negatively regulate intracellular cAMP levels in both epithelial and smooth muscle cells (Li et al., 2007; Sassi et al., 2008). In colonic epithelial cells, Li et al (2007) 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 PDZK1, which serves as a scaffolding protein for MRP4 and CFTR. 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 (Ritter et al., 2005; Russel et al., 2008); however, no one has examined its presence in cardiac myocytes.

CFTR, an anion channel permeable to  $\text{Cl}^-$  and  $\text{HCO}_3^-$ , is most notably studied in epithelial cells, but is also expressed in other cell types, including cardiac myocytes (Lader et al., 2000; Sellers et al., 2010). Activation of CFTR occurs upon ATP-binding and phosphorylation of its regulatory domain by cAMP-, cGMP-, or  $\text{Ca}^{2+}$ -dependent proteins (Gadsby and Nairn, 1999; Ostedgaard et al., 2001; Vaandrager et al., 1997). In cardiac myocytes, it has been postulated that as a  $\text{Cl}^-$  channel CFTR is involved in minimizing the depolarizing effect of  $\text{Ca}^{2+}$  entry upon  $\beta$ -adrenoreceptor stimulation (Duan, 2009).

In the current study we set out to determine if MRP4 is expressed in cardiac myocytes and if so, examine its involvement in cAMP regulation. We used neonatal ventricular myocytes to monitor changes in contraction rate and fluorescence resonance energy transfer (FRET) to follow changes in cAMP during inhibition of MRP4. Our experiments show that MRP4 is expressed in cardiac myocytes and is involved in the regulation of  $\beta$ -adrenergic stimulated cAMP signaling. Unlike other means of cAMP potentiation, such as PDE4 inhibition, inhibition of MRP4 utilizes CFTR, which is involved in mediating  $\beta$ -adrenergic stimulated contraction rate.

## 2. Materials and Methods

### 2. 1. Materials

Isoproterenol and genistein came from Sigma-Aldrich (St. Louis, MO). MK571 (L 660711 or (E)-3-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]][[3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid) was purchased from Cayman Chemical (Ann Arbor, MI). Rolipram was from Calbiochem (San Diego, CA). CFTR<sub>inh</sub>-172 was obtained from Calbiochem or the Cystic Fibrosis Foundation Therapeutics (CFFT) compound

collection (Rosalind Franklin University of Medicine and Science, Chicago, IL). PG-01 was also obtained from the CFFT compound collection. Drugs were made as 1:1000 stock solutions in DMSO or H<sub>2</sub>O (isoproterenol) and diluted the day of the experiment.

## 2. 2. Cardiac myocyte culture

Neonatal ventricular myocytes were cultured from wildtype (FVB),  $\beta_1$ -adrenoceptor knockout (KO) (129/SV, C57B16/J, DBA2/J) (Rohrer et al., 1996),  $\beta_2$ -adrenoceptor knockout (129/SV, FVB/N) (Chruscinski et al., 1999),  $\beta_1/\beta_2$ -adrenoceptor double knockout (cross-mating  $\beta_1$  and  $\beta_2$  KO) (Rohrer et al., 1999), or CFTR KO (strain 2515 B6.129S6-*Cftr<sup>tm1Kth</sup>/J*) mice (Zhou et al., 1994). All procedures with animals were approved by the University of Illinois IACUC. Cells were isolated and cultured using a similar protocol as previously reported (Xiang et al., 2005). Briefly, freshly born mice were sacrificed and 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. 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 h prior to experiments.

## 2. 3. Western blot

Cultured cardiomyocytes were isolated in 1X dulbecco's phosphate buffer solution containing the protease inhibitors PMSF (250  $\mu$ M), NaF (2.5 mM), Na<sub>3</sub>VO<sub>4</sub> (2.5 mM), benzamidine (1 mM) and lysed in PBS with 2% Triton-X 100 and centrifuged prior to protein estimation. For electrophoresis, samples were run out on a 4–15% agarose gel. Following transfer to a PVDF membrane, MRP4 was detected using an IgG anti-MRP4 monoclonal antibody (Li et al., 2007).

## 2. 4. Myocyte contraction rate

Measurement of myocyte contraction rate was performed as previously reported (Xiang et al., 2002). 35 mm dishes were placed in a temperature/CO<sub>2</sub> regulated apparatus allowing for a constant environment of 37° C and 95% O<sub>2</sub>/5% CO<sub>2</sub>. Using a CCD camera and MetaMorph imaging software (Molecular Devices, Downingtown, PA), 5 s video images were obtained every 2 min. Movements in the x and y plane were calculated and number of deflections noted as number of contractions.

## 2. 5. Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) was used to measure real-time changes in intracellular cAMP as previously described (De Arcangelis et al., 2010). Following 24 h in culture, neonatal cardiomyocytes plated at low-density were infected with adenovirus (100 multiplicity of infection, 24 h) containing the cAMP sensor ICUE3 (Soto et al., 2009). Between 24 and 48 h post-infection myocytes were washed and stored in DPBS with 1 mM CaCl and 1 mM MgCl for FRET recordings. Myocytes were imaged with a Zeiss Axiovert 200M microscope fitted with a 40x/1.3 NA oil-immersion objective lens and a CCD camera controlled by MetaFlour software (Molecular Devices, Downingtown, PA). Acquisition was set to 200 millisecond exposure in both channels every 20 s. The binding of cAMP to the Epac site of ICUE3 causes a decrease in FRET (DiPilato et al., 2004). Yellow-to-cyan ratios were calculated at different time points, background corrected, and normalized with data presented as changes in the inverse of YFP/CFP.

## 2. 6. Data analysis

All data are expressed as means  $\pm$  standard error, except where indicated. For comparison between two groups of data, Student's unpaired *t*-test was used to determine significance, while multiple groups were compared by analysis of variance (ANOVA) with Bonferonni post-tests using Prism software (GraphPad, San Diego, CA). Results were considered significant at  $P < 0.05$  and are denoted accordingly. Curve fitting and calculation of  $EC_{50}$  was performed by SigmaPlot (Systat, San Jose, CA).

## 3. Results

### 3. 1. Expression of MRP4 in cardiac myocytes

To determine if MRP4 is involved in cardiac myocyte signaling, we first examined the expression of MRP4 protein in mouse neonatal ventricular myocytes using a specific anti-MRP4 monoclonal antibody. As seen in Fig. 1, this antibody recognized MRP4 in MRP4-transfected HEK cells, but not in untransfected HEK cells. Additionally, in two different isolations, this antibody recognized MRP4 protein in wildtype (WT) neonatal ventricular myocytes indicating that MRP4 protein is expressed in neonatal cardiomyocytes.

### 3. 2. Inhibition of MRP4 potentiates $\beta$ -adrenergic stimulated contraction rate

Subsequently, we examined if MRP4 was functionally active and involved in the regulation of cAMP and  $\beta$ -adrenergic signaling. Similar to De Arcangelis et al (2010) we found that isoproterenol, a non-specific  $\beta$ -adrenoceptor agonist, stimulated dose-dependent increases in cardiomyocyte contraction rate with an  $EC_{50}$  of  $1.4 \times 10^{-8}$  M (Fig. 2A and B). We also found that this response was predominantly mediated by  $\beta_1$ -adrenoceptors (Fig. 2C). Subsequently, we examined the effect of MK571, a pharmacological inhibitor of MRP4, on submaximal ( $10^{-8}$  M) or maximal ( $10^{-5}$  M) isoproterenol-stimulated contraction rate. We found that cardiac myocytes pre-treated with MK571 (40  $\mu$ M) displayed an increased response to submaximal isoproterenol stimulation, compared to DMSO-treated myocytes. This effect was specific to submaximal stimulation, as MK571 had no effect on maximal isoproterenol stimulation (Fig. 3A). This effect of MK571 on contraction rate occurred at 20 and 40  $\mu$ M, but not 10  $\mu$ M (Fig. 3B). To determine if MK571-dependent potentiation of submaximal isoproterenol-stimulated contraction rate was  $\beta$ -adrenoceptor subtype specific we investigated the effect of MK571 in  $\beta$ -adrenoceptor KO mice. As seen in Fig. 3C, MK571 only potentiated submaximal isoproterenol-stimulated contraction rate in  $\beta_2$ -adrenoceptor KO mice, but not  $\beta_1$ - or  $\beta_1/\beta_2$ -adrenoceptor KO mice, indicating that MRP4 inhibition specifically alters  $\beta_1$ -adrenoceptor stimulated contraction rate in neonatal cardiomyocytes.

### 3. 3. Inhibition of MRP4 increases $\beta$ -adrenergic stimulated cAMP

To provide further evidence for MRP4 involvement in  $\beta$ -adrenergic signaling in cardiac myocytes we used FRET to measure real-time changes in intracellular cAMP following isoproterenol stimulation in the presence or absence of MRP4 inhibition. Addition of  $10^{-8}$  M isoproterenol caused a substantial increase in intracellular cAMP that was characterized by an initial rapid, but transient, increase followed by a secondary slow, but steady, increase over time (Fig. 4A). In contrast, stimulation of cardiomyocytes with  $10^{-5}$  M isoproterenol caused a significantly increased amount of cAMP compared to  $10^{-8}$  M, due to potentiation of the secondary cAMP response to stimulation (Fig. 4B). When we simultaneously added MK571 (40  $\mu$ M) and  $10^{-8}$  M isoproterenol, intracellular cAMP significantly increased compared to isoproterenol alone, both in magnitude and duration. In contrast, the level of cAMP activity stimulated by MK571 and  $10^{-5}$  M isoproterenol was similar to  $10^{-5}$  M

isoproterenol alone, indicating that MK571 potentiates cAMP activity stimulated by submaximal, but not maximal isoproterenol stimulation.

### 3. 4. CFTR mediates MRP4-dependent alterations in contraction rate

Based on previous findings that MRP4 activity can influence CFTR activity in epithelial cells (Li et al., 2007), we next examined if MRP4-dependent potentiation of contraction rate involved CFTR in neonatal cardiac myocytes. While we have previously shown that CFTR is involved in un-stimulated neonatal mouse contraction rate (Sellers et al., 2010), there is currently no evidence of its involvement in  $\beta$ -adrenergic stimulated contraction rate. Therefore, in similar experiments to those done in WT mice (Fig. 2), we examined the effect of various doses of isoproterenol on cardiomyocyte contraction rate in CFTR KO mice. Elimination of CFTR expression resulted in a preferential loss of response to isoproterenol stimulation at doses less than  $10^{-7}$  M with no effect on higher doses (Fig. 5A and C). Pharmacological inhibition of CFTR in WT mice with CFTR<sub>inh</sub>-172 (20  $\mu$ M) produced similar results when stimulated with  $10^{-8}$  and  $10^{-5}$  M isoproterenol (Fig. 5C). This inhibitory effect of CFTR<sub>inh</sub>-172 on submaximal isoproterenol stimulation also occurred in  $\beta_2$ -adrenoceptor KO mice (DMSO:  $10.5 \pm 1.5$  vs. CFTR<sub>inh</sub>-172:  $4.7 \pm 1.7$   $\Delta$ beats/min,  $P < 0.05$ ,  $n = 6$  each), confirming involvement of CFTR in  $\beta_1$ -stimulated contraction rate. Conversely, pre-activation of CFTR with genistein (50  $\mu$ M) specifically potentiated contraction rates stimulated with isoproterenol at doses less than  $10^{-7}$  M, with no effect on contraction rate at maximal doses (Fig. 5B and C). These results were confirmed with the CFTR activator PG-01 (Pedemonte et al., 2005) (Fig. 5C). Thus, positive or negative modulation of CFTR preferentially altered contraction rates in response to submaximal isoproterenol stimulation.

When we examined the effect of MK571 in CFTR KO mice we found that MK571 failed to increase submaximal ( $10^{-8}$  M) isoproterenol stimulated contraction rate, such as seen in WT mice, with no effect on maximal ( $10^{-5}$  M) stimulation (Fig. 6A).

### 3. 5. PDE4-dependent increases in contraction rate are CFTR-independent

Rolipram, an inhibitor of PDE4, has been shown to increase cAMP and contraction rates in response to  $10^{-9}$  M isoproterenol (De Arcangelis et al., 2010). Therefore, we examined if rolipram-dependent increases in contraction rate were also CFTR-dependent. We found that in the presence of rolipram  $10^{-8}$  M isoproterenol stimulated a significant increase in contraction rate that was comparable in magnitude to that during maximal stimulation ( $10^{-5}$  M) (Fig. 6B). It was also significantly increased from that of MK571 ( $P < 0.01$ ). In contrast to MK571, in CFTR KO cardiomyocytes, rolipram continued to elicit a significant increase in contraction rate that was similar to that seen in WT mice (Fig. 6B). These results indicate that MK571-induced potentiation of contraction rate is CFTR-dependent, whereas rolipram-induced potentiation is CFTR-independent.

## 4. Discussion

Cardiac myocytes utilize a vast network of membrane-bound and intracellular proteins to regulate contraction. Amongst these,  $\beta$ -adrenoceptors ( $\beta_1$ ,  $\beta_2$ ,  $\beta_3$ ) are central to modulating dynamic changes in cardiomyocyte contraction rate and force generation (Devic et al., 2001). Simplistically,  $\beta_1$ -adrenoceptor activation leads to activation of protein kinase A (PKA) via increased production of cAMP by adenylyl 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 (2008), but include the sarcolemmal L-type  $\text{Ca}^{2+}$  current, the slowly activating component of delayed rectifier  $\text{K}^+$  current, the plasma membrane  $\text{Ca}^{2+}$ -ATPase, the ryanodine receptor (Huke and Bers, 2008), and

phospholemman and phospholamban, which regulate the Na<sup>+</sup>/K<sup>+</sup> ATPase and sarcoplasmic reticulum Ca<sup>2+</sup> pump (SERCA), respectively (Despa et al., 2005).

Of central importance to our study, cAMP and PKA regulation are critical to the generation and regulation of spontaneous beating neonatal cardiac myocytes, as loss of either one of these markedly blunts the ability of these cells to contract (Sellers et al., 2010). Unlike adult ventricular myocytes, neonatal ventricular myocytes have the ability to beat as a single syncytial unit. This property allowed us to examine myocyte function without exogenous perturbation. While it may be tempting to compare these cells to adult pacemaker or atrial cells, which also spontaneously contract, it is more likely that neonatal ventricular myocytes are analogous to adult ventricular myocytes. In a comparison of their electrophysiological properties, Nuss and Marban (1994) showed that mouse neonatal and adult ventricular myocytes express similar ion channels and have comparable action potential durations.

Widely studied as an ATP-dependent drug efflux pump, MRP4 has also been shown to regulate intracellular cAMP levels via transport out of the cell. Our use of the FRET probe ICUE3 shows, for the first time, that MRP4 is involved in negatively regulating cAMP levels in cardiac myocytes. This data is in agreement with prior studies in epithelial (Li et al., 2007) and smooth muscle cells (Sassi et al., 2008) which showed that inhibition of MRP4 with the pharmacological MRP4 inhibitor MK571 (Reid et al., 2003) or siRNA targeting MRP4 caused a decrease in the efflux of cAMP, resulting in accumulation of intracellular cAMP and activation of downstream effectors. In our study we utilized the pharmacological inhibitor MK571 to examine the role of MRP4 in cardiac myocytes. The limitation of this approach is that like many other drugs, MK571 has also been shown to affect other targets, such as MRP1 (Mueller et al., 2008), MRP5 (Reid et al., 2003) and cysteinyl leukotriene receptors (CysLT1) (Lynch et al., 1999). While we did not examine these other targets, we do not anticipate that they confound our results. MRP1 has been implicated in the transport of glutathione (Mueller et al., 2008), thus making it unlikely to be involved in our short-term experiments. MRP5 transports cyclic nucleotides, but with a high affinity for cGMP (Jedlitschky et al., 2000). In contrast to the stimulatory effect of cAMP, cGMP negatively regulates cardiomyocyte contraction through reduction of myofilament Ca<sup>2+</sup> responsiveness. CysLT1 receptors are coupled to G<sub>αq</sub> receptors resulting in mobilization of intracellular Ca<sup>2+</sup>. Thus, one would expect that inhibition of MRP5 or CysLT1 would lead to a decrease in contraction rate through increased negative stimulus or a lack of a positive stimulus, respectively. On the contrary, we found that MK571 caused an increase in the intracellular accumulation of isoproterenol-stimulated cAMP and caused an increase in isoproterenol-stimulated cardiomyocyte contraction rate; both of which are supported by an inhibitory effect of MRP4.

PDE4 is a well-established modulator of cAMP signaling in cardiac myocytes, which we have also shown by the ability of rolipram to potentiate submaximal isoproterenol stimulated contraction rate. These findings are similar to De Arcangelis et al (2010) who examined the effect of rolipram at another submaximal concentration (10<sup>-9</sup> M). When stimulated with submaximal concentrations of isoproterenol, rolipram significantly increases intracellular cAMP by potentiating the magnitude and duration of cAMP (De Arcangelis et al., 2010). These effects are similar to what we found, as inhibition of MRP4 with MK571 increased both the initial and sustained phases of cAMP accumulation during submaximal isoproterenol stimulation, which led to increases in contraction rates. Thus, both PDE4 and MRP4 inhibition increase intracellular cAMP, resulting in downstream changes in contraction rates. Despite these similarities, we found that inhibition of MRP4 elicits its effects in a functionally distinct manner from that of PDE4 inhibition. First, inhibition of PDE4 resulted in a more robust increase in contraction rates than inhibition of MK571. This is not likely due to differences in the magnitude of cAMP generation as we found MK571 to

increase cAMP to maximal levels, similar to what has been found with rolipram (De Arcangelis et al., 2010). While rolipram increases isoproterenol-stimulated contraction rate via  $\beta_1$ - and  $\beta_2$ -adrenoceptors (De Arcangelis et al., 2010; Xiang et al., 2005), we found that MK571 potentiates only  $\beta_1$ -adrenoceptor stimulation. Thus, we cannot rule out that recruitment of additional  $\beta$ -adrenoceptors could account for the difference in contraction rates between MRP4 and PDE4 inhibition. Second, we found that MRP4-dependent potentiation of isoproterenol stimulated contraction rate was dependent on CFTR expression, but PDE4-dependent potentiation was not. With this latter finding, we hypothesize that the differences in contraction rates between PDE4 and MRP4 inhibition occur as a result of their distinct spatiotemporal resolutions, not their absolute magnitude.

Compartmentalization of G-protein coupled receptor complexes and cAMP in response to their activation accounts for distinct functional responses to different stimuli that produce increases in cAMP. De Arcangelis et al (2010) have proposed a model whereby submaximal  $\beta$ -adrenoceptor stimulation leads to localized increases in cAMP, with spread constrained by PDE4. Inhibition of PDE4 or maximal activation of  $\beta$ -adrenoceptors eliminates this “brake” and cAMP spreads and exerts farther-reaching effects. Additionally, others have shown that inhibition of PDE3 leads to spatially confined subsarcolemmal increases in cAMP, whereas inhibition of PDE4 results in broad cytosolic cAMP accumulation (Leroy et al., 2008). While we were not able to measure discrete microdomains of cAMP, in epithelial cells Li et al (2007) showed that inhibition of MRP4 leads to increased cAMP in a spatially confined microdomain at or near the plasma membrane. Therefore, we propose that in cardiac myocytes inhibition of MRP4 leads to an increase in subsarcolemmal cAMP, which accounts for its ability to alter contraction rates in a distinct manner from that of PDE4 (Fig. 7). Further investigation of the way in which modulation of MRP4 activity affects cAMP compartmentalization in cardiac myocytes will lead to an increased understanding of the complex events that occur downstream of G-protein coupled receptor activation.

Another important discovery from our study is the role of CFTR in the regulation of  $\beta$ -adrenergic stimulated contraction of cardiac myocytes. In previous work we found that CFTR is involved in the spontaneous contraction of un-stimulated neonatal ventricular myocytes; however, CFTR loss could be compensated for by activation of  $\text{Ca}^{2+}$ -dependent proteins (Sellers et al., 2010). In the current study, we found that modulation of CFTR alters cardiomyocyte contraction rate in response to  $\beta$ -adrenergic stimulation. Cardiac  $\text{Cl}^-$  channels have generally been thought to be involved in minimizing action potential duration in response to  $\beta$ -adrenoceptor stimulation (Duan, 2009). Genistein, a known activator of CFTR, has been found to potentiate subthreshold isoproterenol activation of cardiac  $\text{Cl}^-$  currents to maximal levels (Hool et al., 1998). Additionally, simulation studies predict that increases in CFTR  $\text{Cl}^-$  current will lead to shortening of the myocyte action potential during  $\beta_1$ -adrenoceptor activation (Kuzumoto et al., 2008). Our findings that pre-activation of CFTR, with genistein or PG-01, increases submaximal isoproterenol stimulated contraction rate support these findings. Interestingly, we found that the effect of CFTR only occurred at submaximal concentrations of isoproterenol ( $<10^{-7}$  M). Based on the evidence presented above, we hypothesize that CFTR is primarily involved in mediating functional effects caused by local changes in cAMP at the sarcolemmal membrane. When cAMP signals are constricted to the plasma membrane, they are confined to a microdomain that contains CFTR, leading to its activation and modulation of myocyte contraction. In contrast, a global increase of cAMP by high concentrations of isoproterenol or inhibition of PDE4 would allow, or increase, phospholamban phosphorylation and activation of other ion channels, including  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels through sarcoplasmic reticulum release of  $\text{Ca}^{2+}$  (De Arcangelis et al., 2010) (Fig. 7). To this effect, preliminary studies show that  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels are important in mediating contraction rate increases stimulated by  $10^{-5}$  M isoproterenol (Z.M. Sellers, unpublished results).

In epithelial cells CFTR is physically and functionally linked to MRP4 through interactions with PDZK1 providing a compartment where MRP4-dependent increases in cAMP can lead to activation of CFTR (Li et al., 2007). Additionally, in lung epithelial cells,  $\beta_2$ -adrenoceptors form a complex with PKA and CFTR through PDZ interactions with ezrinradixinmoesin-binding phosphoprotein 50 (EBP50, also referred to as NHERF) (Naren et al., 2003). He et al (2006) have shown that  $\beta_1$ -adrenoceptors interact with multiple PDZ scaffold proteins in HEK-293 and COS-7 cells, while Valentine and Haggie (2011) found that rat H9c2 cells (derived from embryonic myocardium) utilize PDZ and AKAPs to spatially organize  $\beta_1$ -adrenoceptors. Further work is necessary to explore compartmentalized interactions between  $\beta_1$ -adrenoceptors, CFTR, and MRP4 in cardiac myocytes, however, our finding that MRP4-dependent potentiation of contraction rate is  $\beta_1$ -adrenoceptor-dependent and requires CFTR, supports this notion. Subsequent examination of the role of CFTR in  $\beta$ -adrenergic stimulated heart function will lead to a more complete understanding regarding the physiologic control of heart function.

## 5. Conclusions

This is the first report of MRP4 expression or activity in cardiac myocytes. Likewise, we have also provided the first experimental evidence for involvement of CFTR in  $\beta$ -adrenergic cardiomyocyte contraction. Our findings suggest that both MRP4 and CFTR are involved in regulating and mediating the function of local, compartmentalized increases in cAMP. The involvement of these membrane proteins in  $\beta$ -adrenoceptor stimulated cAMP regulation and function make them important factors to consider in future studies examining the regulation of  $\beta$ -adrenergic signaling and contraction in the heart.

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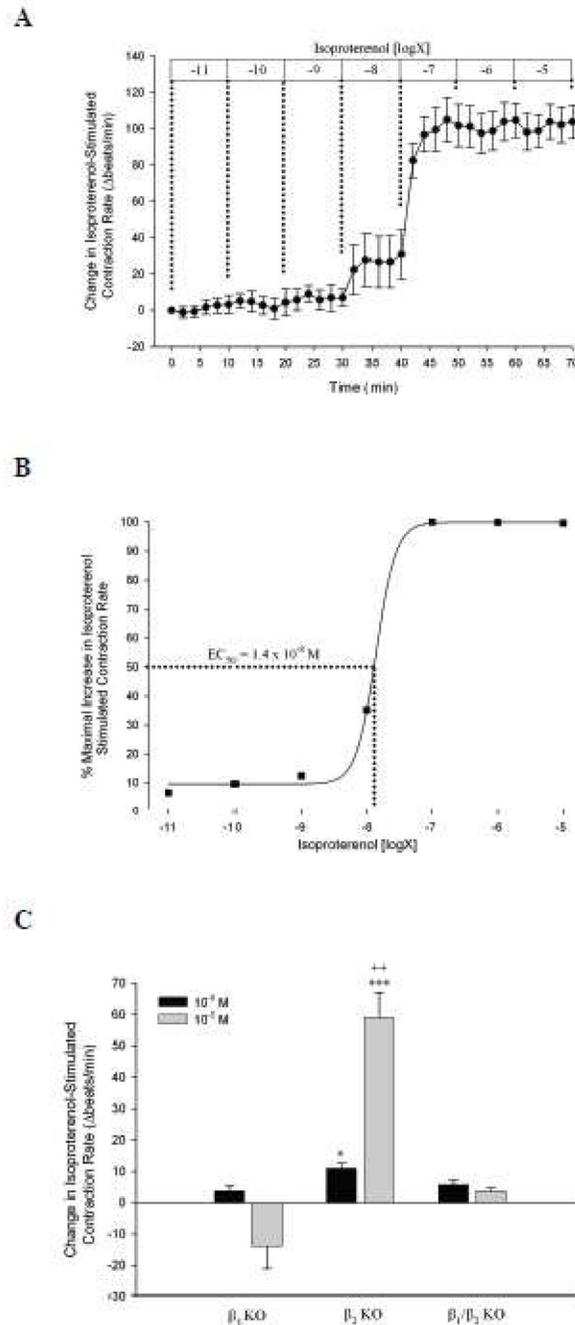
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**Fig. 1. Expression of MRP4 in mouse neonatal ventricular myocytes**

MRP4 protein expression was probed in untransfected HEK cells, MRP4-transfected HEK cells, and two different samples of WT neonatal ventricular myocytes. Cells were cultured and harvested in lysis buffer. 200  $\mu$ g and 40  $\mu$ g of cardiomyocytes or HEK cells, respectively, were loaded into agarose gels and MRP4 expression was detected by a MRP4-specific antibody (1:5000) recognizing MRP4 at approximately 159 kDa.

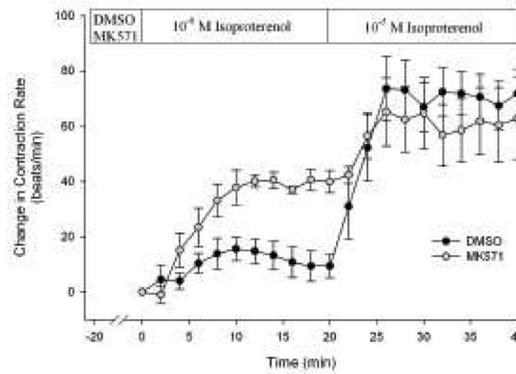


**Fig. 2. Dose-dependent isoproterenol stimulated contraction rate changes in neonatal cardiomyocytes**

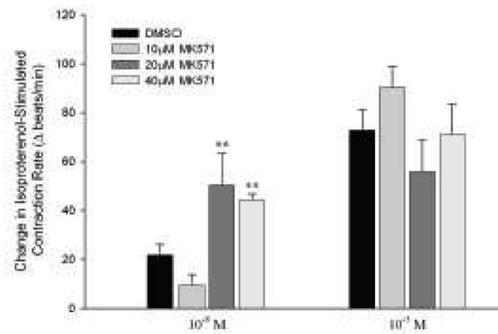
**A.** Spontaneously beating WT neonatal cardiomyocytes ( $n = 6$ ) were exposed to incrementally increasing doses of isoproterenol ( $10^{-11}$  to  $10^{-5}$  M) for 10 min each. Changes in contraction rate from baseline were recorded and plotted. **B.** Maximal changes in contraction rate for each dose of isoproterenol were plotted and curve-fitted with calculation of  $EC_{50}$  via SigmaPlot software. **C.** Following a 10 min baseline, cardiomyocytes from  $\beta_1$ -,  $\beta_2$ -, and  $\beta_1/\beta_2$  KO mice were exposed to  $10^{-8}$  M isoproterenol, then  $10^{-5}$  M isoproterenol for 10 min each. Peak responses to each concentration were subtracted from values prior to

isoproterenol addition. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$  vs.  $\beta_1$ - and  $\beta_1/\beta_2$  KO mice by ANOVA. +,  $P < 0.01$  vs.  $10^{-8}$  M by Student's  $t$ -test.

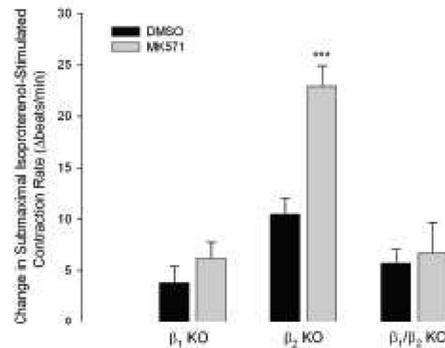
A



B



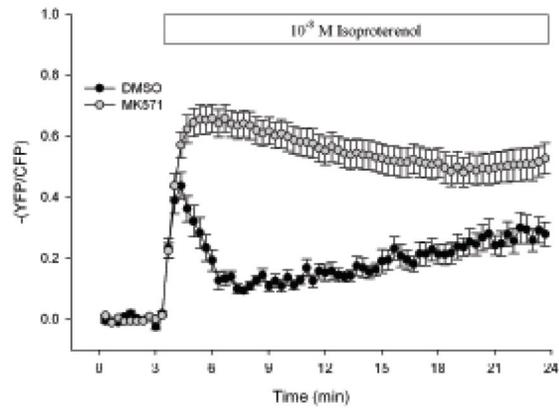
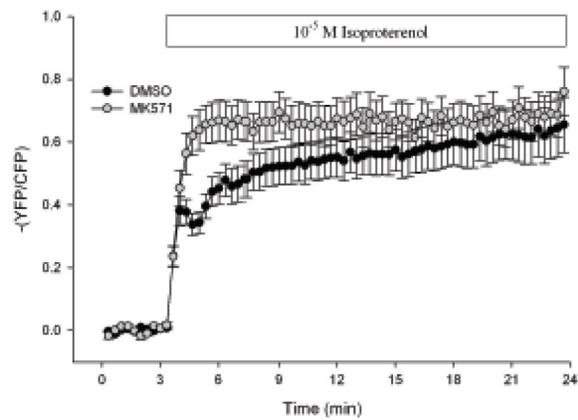
C



**Fig. 3. Pharmacological inhibition of MRP4 by MK571 potentiates submaximal isoproterenol-stimulated contraction rate via  $\beta_1$ -adrenoceptors**

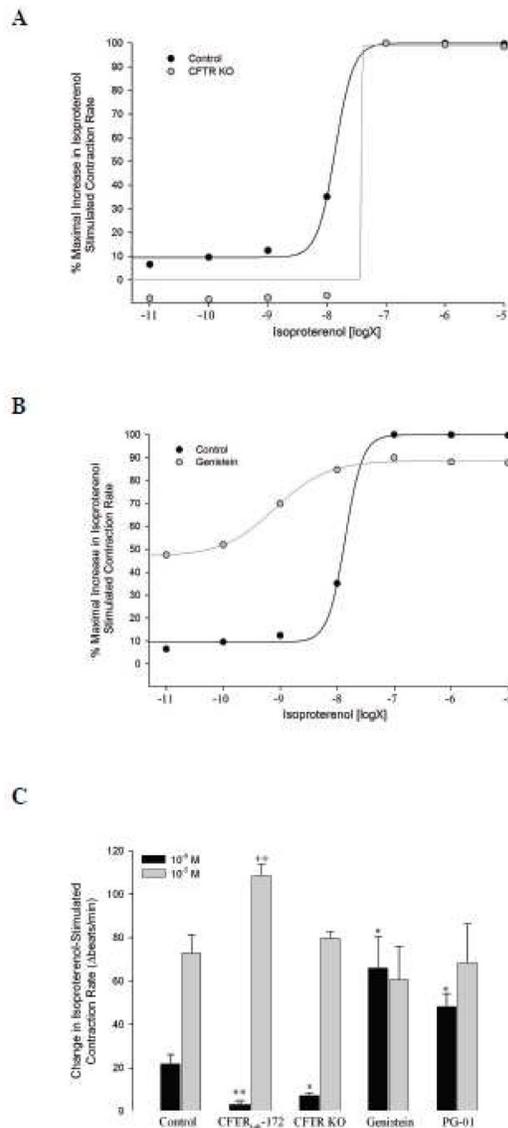
**A.** WT syncytial cardiomyocytes were exposed to DMSO ( $n = 8$ ) or MK571 ( $40 \mu\text{M}$ ,  $n = 5$ ) for 20 min prior to isoproterenol stimulation. Subsequently, myocytes were stimulated with  $10^{-8}$  M, then  $10^{-5}$  M isoproterenol for 20 min each. **B.** Similarly, additional experiments were carried out with 10 or 20  $\mu\text{M}$  MK571 ( $n \geq 5$ ) and peak changes in contraction rates were compared to DMSO. \*\*,  $P < 0.01$  vs. DMSO by Student's  $t$ -test. **C.** Spontaneously beating neonatal ventricular myocytes from  $\beta_1$ -,  $\beta_2$ -, and  $\beta_1/\beta_2$  KO mice were exposed to DMSO ( $n \geq 6$ ) or MK571 ( $40 \mu\text{M}$ ,  $n \geq 6$ ) in a similar manner as above with peak responses

to submaximal isoproterenol stimulation ( $10^{-8}$  M) represented. \*\*\*,  $P < 0.001$  vs. DMSO for each group by Student's *t*-test.

**A****B**

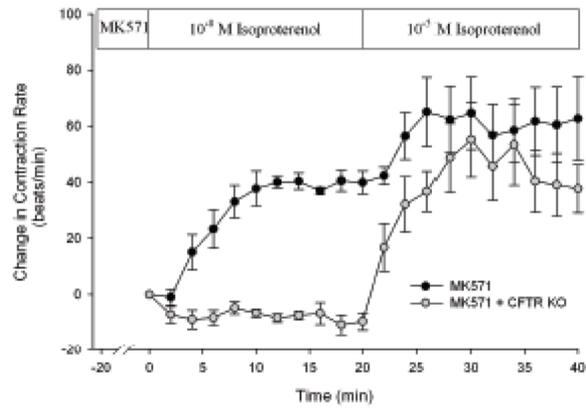
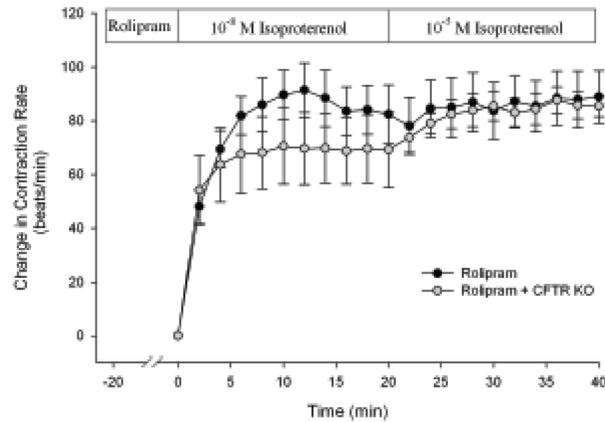
**Fig. 4. MK571 increases intracellular cAMP in response to submaximal, but not maximal, isoproterenol stimulation**

WT cardiomyocytes were cultured at low-density and incubated with an ICUE3 adenovirus for 24–48 h. Myocytes were exposed to DMSO ( $n \geq 20$ ) or MK571 (40  $\mu$ M,  $n \geq 23$ ) for 20 min prior to data acquisition. Intracellular cAMP was measured for a 3 min baseline, after which cells were exposed to  $10^{-8}$  M (A) or  $10^{-5}$  M (B) isoproterenol for 20 min. Data are expressed as changes from initial measurements made during baseline.



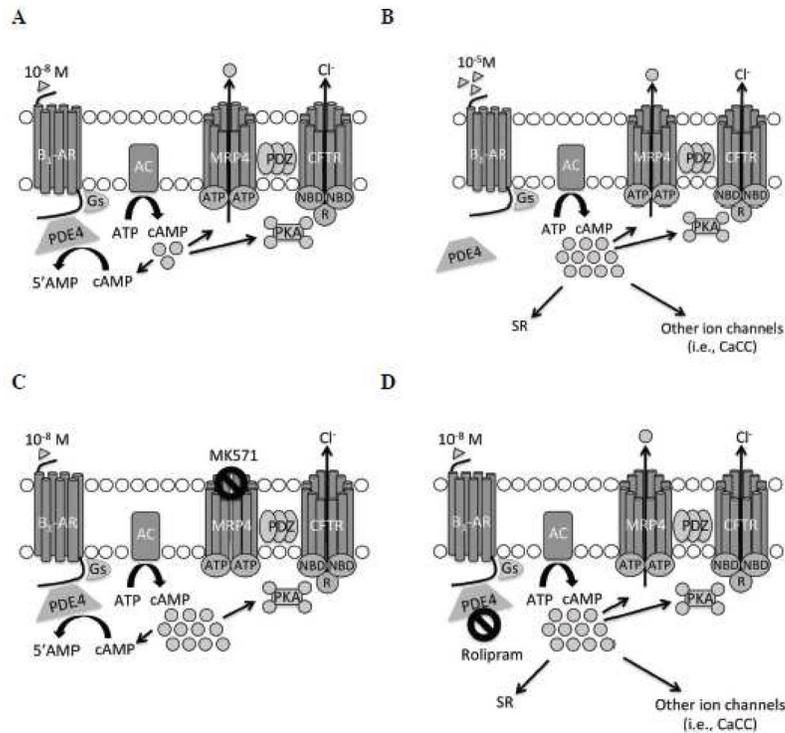
**Fig. 5. Effect of CFTR modulation on isoproterenol stimulated contraction rate**

**A.** Neonatal ventricular myocytes from CFTR KO mice ( $n = 4$ ) were exposed to graded doses of isoproterenol ( $10^{-11}$  to  $10^{-5}$  M) in a similar manner as Fig. 2. Responses were compared to those in WT mice (control, same as Fig. 2). **B.** In a similar manner, WT cardiomyocytes were exposed to genistein ( $50 \mu\text{M}$ ,  $n = 4$ ) for 20 min prior to stimulation with isoproterenol. **C.** Comparison of submaximal ( $10^{-8}$  M) and maximal ( $10^{-5}$  M) isoproterenol stimulated contraction rate responses with CFTR disruption (CFTR KO mice or 20 min pre-incubation with CFTR<sub>inh</sub>-172 ( $20 \mu\text{M}$ ) ( $n \geq 5$  for each)) or pre-activation (20 min pre-incubation with genistein or PG-01 ( $5 \mu\text{M}$ ) ( $n \geq 4$  for each)). Data are expressed as peak responses over baseline. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  vs. control +  $10^{-8}$  M isoproterenol by Student's *t*-test. ++,  $P < 0.01$  vs. control +  $10^{-5}$  M isoproterenol Student's *t*-test.

**A****B**

**Fig. 6. MRP4-, but not PDE4-dependent potentiation of isoproterenol stimulated contraction rate, is CFTR-dependent**

CFTR KO cardiomyocytes were incubated with MK571 (40  $\mu$ M, n = 6, **A**) or rolipram (10  $\mu$ M, n = 8, **B**) for 20 min prior to stimulation with  $10^{-8}$  and  $10^{-5}$  M isoproterenol. Data are presented with data from WT mice (taken from Fig. 3).



**Fig. 7. Proposed involvement of MRP4 and CFTR in  $\beta_1$ -adrenergic stimulation**

**A.**  $10^{-8}$  M isoproterenol stimulation. **B.**  $10^{-5}$  M isoproterenol stimulation. **C.**  $10^{-8}$  M isoproterenol stimulation in the presence of MRP4 inhibition by MK571. **D.**  $10^{-8}$  M isoproterenol stimulation in the presence of PDE4 inhibition by rolipram. Additional abbreviations:  $\beta_1$ -AR,  $\beta_1$ -adrenoceptors; AC, adenylyl cyclase; NBD, nucleotide binding domain of CFTR; R, regulatory domain of CFTR; SR, sarcoplasmic reticulum; CaCC,  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels.