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Title Page

Designing Calcium Release Channel inhibitors with Enhanced Electron Donor Properties: Stabilizing the Closed State of RyR1

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Running Title Page

Running Title: Ryanodine Receptor Inhibitors

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Abbreviations: 4-CmC, 4-chloro-3-methyl phenol or 4-chloro-m-Cresol; 4-MmC, 4-methoxy-3-methyl-phenol, or 4-methoxy-m-Cresol; EGTA, Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; FKBP12, FK506 Binding Protein 12 kilodalton; Hepes, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); Pipes, piperazine-N,N′-bis(2-ethanesulfonic acid); RyR1, Ryanodine Receptor type 1; RyR2, Ryanodine Receptor type 2; SERCA1, Sarcoplasmic/Endoplasmic reticulum Ca^{2+} ATPase type 1; SR, Sarcoplasmic Reticulum; TBST, Tris Buffered Saline Tween-20; XTT, 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt.

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Abstract

New drugs with enhanced electron donor properties that target the ryanodine receptor from skeletal muscle sarcoplasmic reticulum (RyR1) are shown to be potent inhibitors of single channel activity. In this paper we synthesize derivatives of the channel activator 4-chloro-3-methyl phenol (4-CmC) and the 1,4-benzothiazepine channel inhibitor K201 (JTV519) with enhanced electron donor properties. Instead of activating channel activity (~100 μM), 4-CmC's 4-methoxy analog (4-methoxy-3-methyl phenol, 4-MmC) inhibits channel activity at sub-micromolar concentrations (IC_{50} = 0.34 ± 0.08 μM). Increasing the electron donor characteristics of K201, by synthesizing its dioxole congener, results in an approximately 16 times more potent RyR1 inhibitor (IC_{50} = 0.24 ± 0.05 μM) as compared to K201 (IC_{50} = 3.98 ± 0.79 μM). Inhibition is not caused by an increased closed time of the channel, but appears to be caused by an open state block of RyR1. These alterations to chemical structure do not influence the ability of these drugs to affect Ca^{2+} dependent ATPase activity of SERCA1. Moreover, the FKBP12 protein, which stabilizes RyR1 in a closed configuration, is shown to be a strong electron donor. It appears as if FKBP12, K201, its dioxole derivative, and 4-MmC inhibit RyR1 channel activity by virtue of their electron donor characteristics. These results embody strong evidence that designing new drugs that target RyR1 with enhanced electron donor characteristics results in more potent channel inhibitors. This represents a novel approach toward designed new more potent drugs aimed at functionally modifying RyR1 single channel activity.

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Introduction

The sarcoplasmic reticulum (SR) is an internal membrane system that controls the myoplasmic Ca^{2+} concentration and hence controls the contractile state of the muscle cell. A large number of chemically diverse compounds have been shown to either activate or inhibit the SR Ca^{2+} release channel. The common characteristic of most channel activators is their ability to act as electron acceptors, and common to the channel inhibitors is their electron donor characteristics. Moreover, there is a strong correlation between the strength of the electron donor/acceptor, and its potency as a channel inhibitor/activator(Marinov et al., 2007). It occurred to us that this could serve as a basis and direction for developing new drugs targeting the RyR.

4-Chloro-3-methyl phenol (4-CmC) is a disinfectant and preservative that activates ryanodine binding and single channel activity in skeletal and cardiac muscle SR at concentrations ranging from 50-400 μM(Herrmann-Frank et al., 1996). It also inhibits the Ca^{2+} pump protein from SR at low millimolar concentrations(Al-Mousa and Michelangeli, 2009). A large number of derivatives of 4-CmC, most of which are commercially available, have been shown to activate the RyR1 at varying concentrations(Jacobson et al., 2006).

K201 (JTV519) is a benzothiazepine derivative which shows both anti-arrhythmic and cardioprotective properties. These beneficial effects to the heart appear to be caused by its ability to decrease the Ca^{2+} leak mediated by the cardiac ryanodine receptor (RyR2). Yet, it is not specific in targeting the SR. K201 alters the gating of the dihydropyridine receptor (Kohno et al., 2003), inhibits Annexin V dependent Ca^{2+} fluxes(Kaneko et al., 1997), and has a natriuretic effect on the glomerular filtration rate (Lisy and Burnett, 2006). K201 also blocks the delayed rectifying K^+ channel which results in prolongation of the cardiac action potential (Kiriyama et al., 2000).

A substructure of K201, called S107, has been shown to enhance binding of FKBP12.6 to a R2474S mutant form of RyR2, inhibit the Ca^{2+} leak from RyR2 channels, and prevent cardiac arrhythmias. It was also shown that this drug fails to interact with other cardiac ion channels at concentrations up to 10 μM(Lehnart et al., 2008). Moreover, S107 prevents dissociation of the

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FKBP12/RyR1 complex and prevents a decline in exercise performance in skeletal muscle(Bellinger et al., 2008).

Exercise intolerance and skeletal muscle weakness are major limiting factors in humans with chronic heart failure. PKA hyperphosphorylation of RyR1, and the dissociation of the FKBP12/RyR1 complex have been implicated in defects in skeletal muscle intracellular Ca^{2+} handling and early fatigue in heart failure muscle (Wehrens et al., 2005). K201 has been shown to inhibit the reconstituted solubilized RyR1 with an IC_{50} ~ 25 μM and to induce sub-conductance states at positive holding potentials, but not at negative potentials. In permeabilized skeletal muscle fibers, K201 also decreased spark frequency, but increased the frequency of embers(Almassy et al., 2008).

In this study, we design two new derivatives of 4-CmC and K201 with enhanced electron donor properties and demonstrate that both new drugs act as potent inhibitors of RyR1, independent of the absence or presence of FKBP12. Moreover, these new drugs have no significant effect on channel closed time (τ_c). They primarily inhibit channel activity by decreasing the open time (τ_o) of the channel.

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Materials and Methods

Sarcoplasmic Reticulum Preparation: SR vesicles were isolated from rabbit fast twitch skeletal muscle according to the method of MacLennan(MacLennan, 1970) with small modifications, 50 μM dithiothreitol and 0.2 μg/ml leupeptin were added to all buffers except the final resuspension buffer. SR vesicles were then further fractionated on a discontinuous sucrose gradient (Salama and Abramson, 1984). The following sucrose solutions (percent by weight) plus 10 mM Hepes, pH 7.0 were layered sequentially in a SW28 centrifuge tube (Beckman): 4 ml of 45%, 7 ml of 40%, 12 ml of 35%, 7 ml of 30%, and 4 ml of 27%. Thirty milligrams of unfractionated SR were layered on top of the gradient and then spun at 22,000 rpm overnight. The heavy fraction (HSR) was used in all single channel experiments. All SR was stored in liquid N_2 immediately following its preparation. All animal care and use protocols were approved by the Portland State University IACUC committee.

ATPase activity – Ca^{2+} dependent ATPase activity of SR vesicles in the presence of the Ca^{2+} ionophore A23187 was determined spectrophotometrically in the presence of varying concentrations of drugs. The standard assay medium contained 100 mM KCl, 20 mM Hepes, 1 mM $MgCl_2$, 1 mM EGTA, 500 μM NADH, 1 mM phosphoenol pyruvate, 5 units of lactate dehydrogenase, and 0.5 mM MgATP at pH 7.0. The rate of NADH oxidation (equivalent to the rate of ATP hydrolysis) was monitored in a stepwise fashion by recording the decrease in absorbance at 340 nm as a function of time as previously described (Favero et al., 1995). The reaction was initiated by the addition of 0.1 mg/ml SR to the standard assay medium containing 1.0 mM EGTA (Ca^{2+} independent ATPase activity). ATPase activity was then recorded following the subsequent addition of $CaCl_2$ to yield a free Ca^{2+} concentration of 8 μM, and the Ca^{2+} ionophore A23187 (1μg/ml). Ca^{2+} independent ATPase activity was subtracted from total activity (typically 20% of total activity) to yield Ca^{2+} dependent ATPase activity.

The FKBP12 protein was over expressed in E. coli BL21(DE3) cells and purified by Ni^{2+}-agarose affinity chromatography using standard protocols(Van Lanen et al., 2005).

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Planar lipid membranes - Bilayers were made from a mixture of 5:3:2 PE:PS:PC (Avanti) painted across a 150 μm whole separating two compartments. The *cis* chamber to which 5-10 μg/ml SR was added contained 400 mM $Cs^+CH_3O_3S^-$, 20 mM Hepes, pH 7.4, while the *trans* side of the membrane contained 40 mM $Cs^+CH_3O_3S^-$, 20 mM Hepes, pH 7.4. Following fusion of an SR vesicle to the bilayer, 4 M $Cs^+CH_3O_3S^-$, 20 mM Hepes, pH 7.4 was added to the trans side of the membrane to equalize the salt concentration on the two sides of the membrane to 400 mM. Channel output was filtered at 800-1000 Hz and traces of not less than three minutes were recorded following addition of various concentrations of channel modulators. Under these filtering conditions, channel open and closed times can be resolved down to ~ 0.5 ms. The average open time of our control single channel recordings was 0.6 ms. Given these experimental conditions, we were not able to directly measure decreases in τ_o. However,

(1) $P_o = T_o/(T_o + T_c)$, where P_o is the open probability, T_o is the total open time, and T_c is the total closed time. Moreover, $T_o = N\tau_o$, and $T_c = N\tau_c$, where N = the number of channel openings = the number of channel closings, τ_o is the average open time, and τ_c is the average closed time. Therefore:

(2) $P_o = \tau_o/(\tau_o + \tau_c)$, and

(3) $\tau_o = P_o\, \tau_c / (1 - P_o)$.

By measuring P_o and τ_c, one can calculate the corresponding value of τ_o. If a channel inhibitor has no significant effect on τ_c, as P_o decreases, τ_o must decrease. All single channel analysis was carried out using the ClampFit program from Axon Instruments pClamp software suite. Changes in τ_c were directly measured, and the corresponding changes in τ_o were calculated under conditions in which P_o had not decreased to < 50% of control. Changes in τ_c and P_o were considered significant using a Student's t-test if the p-value was less than 0.05.

Electron donor measurements were carried out as described by Marinov et al. (Marinov et al., 2007). All measurements were carried out under continuous illumination by visible light in a buffer containing 1 mM Tris, 10 μM methylene blue, 100 μM XTT, pH 7.4 at room temperature. Visible light excited the dye (methylene blue), which led to the sequential formation of a singlet then a triplet state.

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Two triplets combined to form a dye anion and dye cation radical pair. Electron donors donated electrons to the dye cation radical and decrease their concentration. This increased the lifetime of the dye anion radical. In the presence of O_2, the dye anion radical reduced O_2, which yielded superoxide. XTT is a probe that readily reacts with superoxide(Sutherland and Learmonth, 1997). Its reduction by superoxide resulted in a time dependent increase absorbance at 470 nm(Olojo et al., 2005). More potent electron donors increased the rate and amount of reduction of XTT.

The electron acceptor properties of a compound were assayed in the presence of a sacrificial electron donor (i.e 100 μM EGTA). Continuous illumination of methylene blue in the presence of EGTA resulted in the time dependent production of superoxide and a large time dependent reduction of XTT. In the presence of an increasing concentration of an electron acceptor, the rate of reduction of O_2 to form superoxide decreased and the XTT signal (Abs_{470nm}) decreased. The initial rate of reduction of XTT (in the presence of 100 μM EGTA) was plotted vs. the concentration of the electron acceptor and the data were fit to a four parameter logistic curve (SigmaPlot ver. 10). The IC_{50} derived corresponds to the concentration of the electron acceptor, at which half maximal donor activity (referenced to EGTA) was observed.

Assay for dissociation of the FKBP12/RyR1 complex: SR vesicles (1.0mg/ml) in buffer containing 250 mM KCl, 15 mM NaCl, 20 mM Pipes, pH 7.1, 50 μM $CaCl_2$ were incubated with 30μM FK506, or 30μM FK506 + 20 μM K201, or with an equivalent amount of DMSO as a control for 30 min. at 37^{o}C. Samples were then pelleted at 167,000 g for 20 min. in a Beckman Airfuge. The supernatant and pellets were brought up to equivalent volumes. Samples were then solubilized in non-reducing sample buffer containing 62.5 mM Tris, pH 6.8, 10% sucrose, 4% SDS, and 0.02% bromphenol blue, and run onto a 4-12% SDS-PAGE gradient gel. Proteins were then electrophoretically transferred onto PVDF membranes at a constant 15V for 60 min. using a Bio-Rad Trans-Blot Semi-Dry Transfer Cell Apparatus. Membranes were incubated for 1 hr. in blocking buffer (5% nonfat dry milk in TBST) at 23^{o}C with agitation. They were then incubated overnight with a monoclonal antibody to FKBP12 (MAB3777 – R&D Systems, Minneapolis, Minn.) at 1μg/ml in TBST. Membranes were then washed three times for 10 min. in TBST and incubated for 2 hrs. with an anti-rat IgG horse radish peroxidase linked secondary antibody at a 1:10,000 dilution (HAF005 - R&D Systems). Secondary antibody was detected using

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Thermo Scientific SuperSignal West Pico luminescent kit. Western Blot imaging and integration was carried out using an Alpha Innotech Fluorchem SP system (Quansys Biosciences).

Preparation of K201, K201 dioxole derivative and 4-methoxy-3-methyl phenol: K201 was prepared according to the procedure reported by Wehrens and co-workers(Wehrens et al., 2004). The dioxole derivative of K201 (**3**) was synthesized as shown in Scheme 1, following the same procedure as for K201, but modifying the last step where now the brominated thiazepine (**1**) reacts with commercially available 4-benzo[1,3]dioxol-5-ylmethyl-piperidine (**2**). 3-(4-(benzo[d][1,3]dioxol-5-ylmethyl)piperidin-1-yl)-1-(7-methoxy-2,3-dihydrobenzo[f] [1,4]thiazepin-4(5H)-yl)propan-1-one (**3**): The reaction mixture of **1** (0.100 g), 4-benzo[1,3]dioxol-5-ylmethyl-piperidine (**2**, 0.086 g) and Na_2CO_3 (0.086 g) in DMF (5 ml) was stirred at 60 °C for 12 h. DMF was evaporated under vacuum and the product extracted with EtOAc, washed with H_2O and dried over anhydrous Na_2SO_4. After filtration and removal of solvents, the crude product was purified by column chromatography. ^{1}H NMR (400 mHz, $CDCl_3$) δ (ppm): 1.11-2.95 (17H, m), 3.78 (3H, s), 3.92-4.15 (2H, m), 4.70 (2H, s), 5.92 (2H, s), 6.57-7.50 (6H, m). ^{1}H NMR spectra were acquired on a ARX-400 Advance spectrometer (Bruker).

4-MmC was prepared following the procedure reported by Higgins (Higgins et al., 2001) and coworkers starting from 3-methyl-*p*-anisaldehyde. Purification of the product was achieved by flash chromatography and confirmed by ^{1}H NMR. The structure of 4-MmC is shown in the inset of scheme 1.

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Results

We have previously shown that activators of the RyR from sarcoplasmic reticulum are electron acceptors while RyR inhibitors are electron donors. Moreover, more potent channel activators are stronger electron acceptors and more potent channel inhibitors are stronger electron donors (Marinov et al., 2007). In figure 1, we demonstrate that 4-chloro-3-methyl phenol (4-CmC) is an electron acceptor. In figure 1A, 100 μM EGTA is used as a sacrificial electron donor. As described in the Material and Methods section, illumination of methylene blue with visible light, results in the production of anion/cation radical pairs. EGTA readily donates electrons to cation radicals, which increases the lifetime of the dye anion radical. The dye anion radical passes electrons to O_2 which results in the formation of superoxide ($O_2^{\cdot-}$). When XTT is reduced by superoxide, the absorbance at 470 nm increases(Sutherland and Learmonth, 1997). In the presence of an electron acceptor, such as 4-CmC, the dye anion radical passes electrons to the electron acceptor and the amount of superoxide generated decreases. The time dependent reduction of XTT was fit to an exponential rise to a maximum value ($y = A_1*(1-e^{-k_1 t})+ A_2*(1-e^{-k_2 t})$) (fig 1A), and the initial rate of reduction of XTT was determined by the product of $k_1*A_1 + k_2*A_2$. In fig 1B, the decrease in the initial rate of reduction of XTT is plotted vs. [4-CmC] and fit to a four parameter logistic curve. The IC_{50} derived, which corresponds to the concentration of 4-CmC, at which half maximal electron acceptor activity is observed = 21.2 ± 2.0 μM. This value is similar to the concentration of 4-CmC at which the RyR1 is half maximally activated (Herrmann-Frank et al., 1996).

In figure 2 it is shown that replacement of the chloro in position 4 with a methoxy group, converts 4-CmC from an electron acceptor (fig.1) into 4-methoxy-3-methyl phenol (4-MmC), which acts as a potent electron donor. A comparison of the electron donor properties of several relevant compounds at 50 μM is shown in fig 2. The same order of electron donor effectiveness (FKBP12 > 4-MmC> dioxole derivative of K201> K201) was observed at lower concentrations tested (10 μM). Moreover, as shown in fig 3, 4-MmC is a potent inhibitor of single channel activity of RyR1. Fig 3A shows representative single

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channel traces as a function of 4-MmC concentration, while fig. 3B shows the normalized P_o as a function of concentration added to the cis chamber of the bilayer. The calculated IC_{50} for inhibition of single channel activity by 4-MmC is 0.34 ± 0.08 μM. Conversion of an arene such as 4-CmC from an electron acceptor into an electron donor results in the conversion of a channel activator into a potent channel inhibitor.

It has recently been shown that the two RyR2 inhibitors, tetracaine and flecainide differ in their mode of action. While tetracaine inhibits channel activity by increasing channel closed time, flecaininde decreases channel open time, while having little effect on the average closed time of RyR2(Hilliard et al., 2010; Watanabe et al., 2009). In figure 3C, we show that 4-MmC, in a manner similar to flecainide, shows no significant effect on τ_{closed} of RyR1 at concentrations as high as 1.0 μM (where P_o has decreased to ~ 30% of control). Under these conditions, using equation (3),τ_o decreases to 16% of the control value before addition of 4-MmC.

A similar approach toward developing new drugs with enhanced electron donor properties targeting RyR1 focused on the drug K201. K201 and its dioxole derivative were synthesized as described in Material and Methods. As expected, the dioxole derivative of K201 is a more potent electron donor than is K201 (fig. 2). Moreover, as shown in fig. 4, representative traces at the single channel level (4A,4B) and a plot of the normalized P_o versus concentration (4C) demonstrate that the dioxole derivative of K201 (IC_{50} = 0.24 ± 0.05 μM) is approximately 16 times more potent in closing down RyR1 than is K201 (IC_{50} = 3.98 ± 0.79 μM). In a manner similar to 4-MmC, both K201 (fig. 4D) and its dioxole derivative (fig. 4E), decrease RyR1 open probability without significantly increasing the closed time of the channel.

The proposal that K201 stabilizes the RyR by causing the reassociation of the FKBP12/RyR1 complex in skeletal muscle and the FKBP12.6/RyR2 complex in cardiac muscle(Marx et al., 2000) has been challenged by a number of laboratories (Blayney et al., 2010; Hunt et al., 2007; Yano et al., 2003). In figures 5 and 6, it is shown that the addition of 30 μM FK506 results in complete dissociation of the

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FKBP12/RyR1 complex (fig. 5). The FKBP12 protein moves from the pellet fraction in the control (lane 2) to the supernatant fraction (lane 3) following treatment with 30 μM FK506. Addition of 30 μM FK506 + 20 μM K201 does not result in reassociation of the RyR1/FKBP12 complex. The FKBP12 remains in the supernatant fraction (lane 5). At the single channel level, the addition of 30 μM FK506 causes a four to eight fold stimulation of the channel P_o (fig. 6), yet the K_i for inhibition of the normalized single channel activity in the absence (K_i = 3.98±0.79μM) or presence of FK506 (4.6±0.43μM) is unchanged. Inhibition of channel activity by K201 is not affected by the presence or absence of FKBP12.

The specificity of new drugs targeting the RyR is often an important issue in drug development. The non-specific interactions between K201 and multiple targets have limited its clinical usefulness. In fig. 7 it is shown that K201 and its dioxole derivative inhibit the Ca^{2+} ATPase activity of SERCA1 at similar concentrations. The IC_{50} for K201 = 54.8 ± 12.9 μM, while the IC_{50} for the dioxole derivative of K201 = 48.1 ± 7.0 μM (mean ± SE). The enhanced electron donor properties of the dioxole derivative impacts the potency of this drug to inhibit the RyR1 but it does not affect the potency of this drug to inhibit Ca^{2+} ATPase activity. Moreover, as can be seen in fig 7, 4-MmC has no effect on ATPase activity at concentrations as high as 0.5 mM.

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Discussion

In this study we synthesized two new potent RyR1 inhibitors which are derivatives of the known RyR1 modulators, 4-chloro-3-methyl phenol (4-CmC) and K201. 4-CmC activates RyR1 at a concentration range from 50-400 μM(Herrmann-Frank et al., 1996). By replacing the chloro in the 4 position with a methoxy group, we have converted an electron acceptor (fig. 1) into an electron donor (fig. 2) and converted a channel activator into a potent channel inhibitor (fig. 3). In a similar manner, synthesizing the dioxole derivative of K201 converts a RyR1 inhibitor into a more potent electron donor and significantly increases its effectiveness as a channel inhibitor. This non-traditional approach toward drug design was motivated by our earlier observations that all RyR activators tested were electron acceptors, while channel inhibitors were electron donors. Moreover, there was a strong correlation between the effectiveness of these drugs and their potency as either electron donors (inhibitors) or acceptors (activators) (Marinov et al., 2007).

K201 is a strong electron donor (fig. 2) which inhibits both the skeletal (Almassy et al., 2008) and cardiac muscle RyR(Kohno et al., 2003; Wehrens et al., 2004). It has been shown to have both anti-arrhythmic and cardioprotective properties. These beneficial effects on the heart appear to be caused by its ability to decrease the "Ca^{2+} leak" mediated by the cardiac ryanodine receptor (RyR2). It has also been shown that K201 decreases single channel activity of the reconstituted RyR1 isolated from rat skeletal muscle SR (IC_{50} ~ 25μM) (Almassy et al., 2008). Moreover, sub-conductance states of RyR1 were observed at low concentrations of K201, which were associated with enhanced Ca^{2+} fluxes across actively loaded SR vesicles (Almassy et al., 2008). There are several differences between the K201 data described in our study and the previous work carried out by Almassy et al (Almassy et al., 2008). All of the bilayer reconstitution work described in this study was done with rabbit fast twitch muscle SR vesicles which were fused with an artificial membrane as described in Material and Methods. The SR vesicles were not exposed to detergent. In the Almassy et al paper, SR vesicles were isolated from rat skeletal muscle and

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then fractionated on a sucrose gradient in the presence of the non-ionic detergent 3-[(3-cholamido-propyl) dimethylammonio]- 1-propane-sulfonate (CHAPS) before reconstitution in a similar planar bilayer membrane. The IC_{50} for inhibition of single channel activity by K201 is ~ 6 times lower in the present study, and in none of our studies did we observe K201 induced sub-conductance states as described by Almassy et al (Almassy et al., 2008). The RyR1 is a large multi-protein complex. A number of associated proteins have been shown to functionally regulate activity. It is likely that breaking up of this complex by addition of CHAPS alters the sensitivity to inhibition by K201 and results in sub-conductance states such as occur when FKBP12 dissociates from the RyR1(Ahern et al., 1997).

It has been shown by Marks' group that K201 stabilizes the closed state of both RyR1 and RyR2 by increasing the binding of FKBP12 to RyR1 and FKBP12.6 to RyR2 in a heart failure model(Wehrens et al., 2005). This interpretation has been challenged by the observations that K201 almost completely inhibits the Ca^{2+} leak from normal dog heart SR under conditions in which the FKBP12.6 has completely dissociated from RyR2 (i.e. in the presence of 30 μM FK506)(Yano et al., 2003). Moreover, K201 inhibits store-overload-induced Ca^{2+} release (SOICR) in HEK-293 cells expressing RyR2 either with or without FKBP12.6(Hunt et al., 2007). In contrast to the initial hypothesis that K201 stabilizes the RyR by increasing the binding of the FKBP protein, it has recently been shown, using surface plasmon resonance techniques, that K201 in the closed conformation of RyR1/RyR2 reduces the binding affinity of the FKBP12/RyR1 and FKBP12.6/RyR2 complex(Blayney et al., 2010). It does not strengthen this interaction as originally postulated. Although 30 μM FK506 increases the channel open probability ~ four to eight fold (fig 6), and completely displaces FKBP12 from RyR1 (fig. 5), the potency of K201 as a RyR1 inhibitor is unaffected by the presence or absence of the associated FKBP12 (fig 6B). Moreover, RyR1 does not reassociate with FKBP12 in the presence of 20μM K201 – a concentration that significantly inhibits single channel activity (fig. 6). This result does not address the issue of whether or not K201 causes the FKBP12/RyR complex to reassociate in a heart failure model, but it does demonstrate that inhibition of RyR1 by K201 is independent of the presence or absence of FKBP12.

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In an alternative model, Yamamoto and Ikemoto (Yamamoto and Ikemoto, 2002) have proposed that the closed state of the RyR is stabilized by interactions between the N-terminal and central domain of the RyR, and that mutations to the RyR2 lead to 'unzipping' of these two domains result in a Ca^{2+} leak. The binding of K201 to domain$^{2114\text{-}2149}$ has been proposed to correct defective interdomain interactions within RyR2 and decrease the Ca^{2+} leak associated with the failing heart (Yamamoto and Ikemoto, 2002). Mutations to $Gln^{4020}Lys^{4021}$ on RyR1 (corresponding to amino acids 3976-7 in RyR2) abolishes channel activation by 4-CmC(Fessenden et al., 2006). If 4-MmC binds to the same region of RyR1 as 4-CmC, then the inhibitory action of 4-MmC is remote from the interdomain region and the action of 4-MmC is unlikely to involve the direct stabilization or "zipping" of the RyR1 as proposed for the action of K201. Moreover, it is difficult to understand how activation of channel activity by 4-CmC might result in unzipping of the N-terminal and central core domains, while binding of 4-MmC results in domain zipping, unless the domain interactions are sensitive to the electron acceptor/donor properties of these compounds that appear to bind in a region distant from interdomain interaction.

It is well established that thiol reducing agents inhibit RyR1, while thiol oxidizing agents activate the release channel. Reduction of endogenous disulfides on the RyR1 inhibits the flux of Ca^{2+} across the SR(Trimm et al., 1986), channel activity and high affinity ryanodine binding(Zable et al., 1997). The data presented in this paper show that increasing the electron donor properties of drugs targeting the RyR1 results in new potent inhibitors of channel activity. Moreover, the role of electron donors in closing down RyR1 appears to be evident at the level of protein/protein interactions. As shown in figure 2, the purified FKBP12 protein is a more potent electron donor than is K201, its dioxole derivative, or 4-MmC. This suggests that FKBP12 stabilizes RyR1 in its closed configuration by virtue of its electron donor properties. Removal or decreased binding of FKBP12 to RyR1 results in a Ca^{2+} leak which can be reversed by addition of any of a number of drugs which act as strong electron donors. The redox activity of drugs and proteins that interact with the Ca^{2+} release channel is a key factor in modifying function of the SR. The observation that novel drugs with enhanced electron donor properties act as more potent

inhibitors of RyR1 supports the role of redox reactions in channel gating, and presents a new methodology for designing new more potent channel inhibitors.

In a previous publication, we proposed that non-thiol electron acceptors or donors form a reversible charge transfer complex upon binding to RyR1 (Marinov et al., 2007). Channel activators (electron acceptors) were shown to shift the redox potential of RyR1 to more negative redox potentials (RyR1 oxidizes more readily), and the thiol/disulfide balance of the receptor shifts to a more oxidized state. In contrast, channel inhibitors (electron donors) shift the redox potential of the RyR to a more positive potential and the thiol/disulfide balance shifts to a more reduced state (the SH/S-S ratio increases)(Marinov et al., 2007). Consistent with the formation of a charge transfer complex, we have observed that the inhibitory effects of the dioxole derivative of K201 at the single channel level are reversible (not shown). Changing solutions following addition of the dioxole derivative restores channel activity.

The approach taken toward designing new drugs with enhanced electron donor properties has resulted in the development of new more potent inhibitors of the sarcoplasmic reticulum Ca^{2+} release channel. It is not yet known whether a similar approach can be applied toward other protein targets. Previous studies have shown that inhibitors of the L-type Ca^{2+} channel (i.e. nifedipine, verapamil and diltiazem) are electron donors(Marinov and Saxon, 1985), while local anesthetics, antiarrhthmics and some anticonvulsants, which inhibit Na^{+} channels are also electron donors(Marinov, 1985). In contrast, both K201 and its dioxole derivative, with enhanced electron donor properties, show a similar potency toward inhibiting ATPase activity of SERCA1.

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Authorship Contributions

Participated in research design: Ye, Yaeger, Owen, Escobedo, Wang, Singer, Strongin, Abramson
Conducted Experiments: Ye, Yaeger, Owen, Escobedo, Wang, Singer, Strongin, Abramson
Contributed new reagents or analytic tools: Escobedo, Wang, Strongin
Performed data analysis: Ye, Yaeger, Owen, Escobedo, Wang, Singer, Strongin, Abramson
Wrote or contributed to the writing of the manuscript: Ye, Owen, Escobedo, Singer, Strongin, Abramson

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Footnotes

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Conflict of Interest: JJA and RMS are founding members of ELEX Biotech LLC, a start-up company that is developing drugs targeting RyR1 and RyR2.

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Legends for Schemes

Scheme 1. Synthesis of the dioxole derivative of K201. Inset shows the structure of 4-MmC.

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Legends for Figures

Figure 1. 4-chloro-3-methyl phenol is an electron acceptor. (A) Time dependent reduction of XTT in the presence of 100 μM EGTA (●) + the following concentrations of 4-CmC - 5μM (○), 10μM (▼), 20μM (Δ), 50μM (■), 100μM (□), or 200μM (♦). (B) Initial rate of XTT reduction vs. [4-CmC] – data derived from A (Data shown is mean ± SE (n=3)).

Figure 2. Comparison of electron donor properties of control – no addition (●), 50 μM K201(○), 50 μM dioxole derivative of K201(▼), 50 μM 4-MmC(Δ), and 50 μM FKBP12(■). All time dependent scans were repeated at least 3 times under continuous illumination. Values shown are mean ± S.E (n=3). Curves shown are best fits to an exponential rise to maximum.

Figure 3. RyR1 is inhibited by 4-MmC. (A) Representative single channel traces as a function of [4-MmC] – 400 mM $CsCH_3O_3S$, 20 mM Hepes, pH 7.4 on both sides of the bilayer, 50 μM Ca^{2+} *cis* at a holding potential of - 36 mV. Channel openings are shown as downward deflections (C- closed state, O- open state). (B) Open probability normalized to 1.0 vs. [4-MmC] (●). (C) Closed time histogram normalized to 1 vs. [4-MmC]. Data shown is mean ± SE (n=11). Data are expressed relative to 0 μM 4-MmC with $P_o = 0.27 \pm 0.06$, $\tau_{closed} = 1.22 \pm 0.44$ ms.

Figure 4. Dioxole derivative of K201 is a more potent inhibitor of the RyR1 than is K201. (A) Representative single channel traces as a function of [K201]. (B) Single channel traces as a function of [dioxole derivative of K201]. (C) Open probability normalized to 1.0 for control is plotted vs. [K201] (●) and vs. the concentration of the dioxole derivative of K201(○). (D) Closed time histogram normalized to 1 vs. [K201], and (E) vs. [Dioxole K201]. Data shown is mean ± SE (n=8). (D) Data are expressed relative to 0 μM K201 with $P_o = 0.11 \pm 0.06$, $\tau_{closed} = 4.50 \pm 1.37$ ms. (E) Data are expressed relative to 0

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μM dioxole K201 with $P_o = 0.05 \pm 0.02$, $\tau_{closed} = 1.31 \pm 0.33$ ms. Experimental conditions are identical to those described in fig. 3 caption.

Figure 5. FK506 dissociates the FKBP12/RyR1 complex independent of K201. SR vesicles are treated with 30 μM FK506 ± 20 μM K201. Pellets and supernatants are run on a 4-12% polyacrylamide gel, transferred to PVDF and treated with a monoclonal antibody to FKBP12, and a horse radish peroxidase secondary antibody as described in Material and Methods. Lanes 1 and 2 - control; lanes 3 and 4 - treated with 30 μM FK506; lanes 5 and 6 - treated with 30 μM FK506 + 20 μM K201. Lanes 1,3, and 5 are supernatant fractions. Lanes 2,4, and 6 are pellet fractions. Normalized integrated densities are as follows: Lane (1) = 0.00; lane (2) = 1.00; lane (3) = 1.02; lane (4) = 0.01; lane (5) = 1.06; lane (6) = 0.00. This transfer was repeated three times with identical findings.

Figure 6. K201 inhibition of single channel activity is independent of FKBP12. (A) Representative single channel traces of control, following treatment with 30 μM FK506, and following the addition of K201at increasing concentrations. (B)Normalized open probability vs. [K201] in the absence ((●), n=7), and presence of 30μM FK506 ((○), n=6). Data shown are mean ± SE. Open probability of control (no K201) = 0.039 ± 0.011 (n=13), and in the presence of FK506 = 0.154 ± 0.045 (n=7). Experimental conditions are identical to those described in fig. 3 caption.

Figure 7. Ca^{2+} dependent ATPase activity vs. concentration of K201 (●), the dioxole derivative (○), and 4-methoxy-3-methyl phenol (▼). Data shown is mean ± SE (n=3).

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Scheme 1

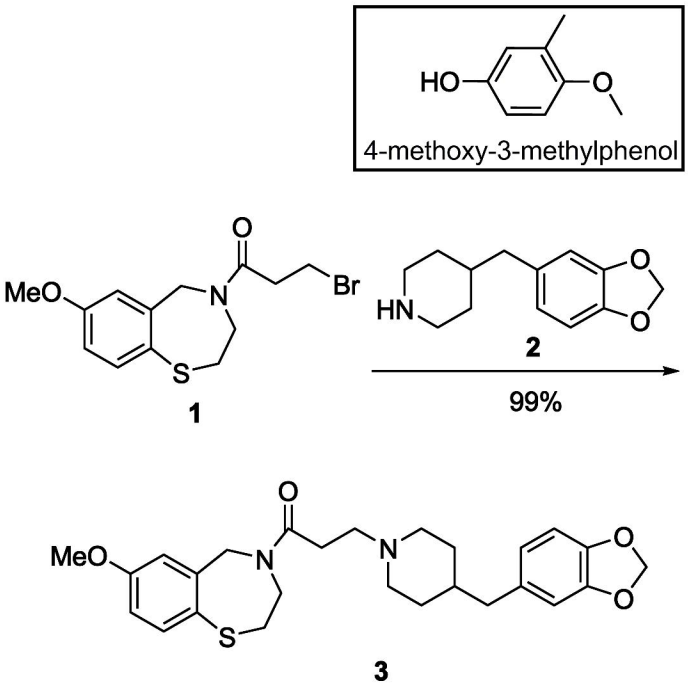
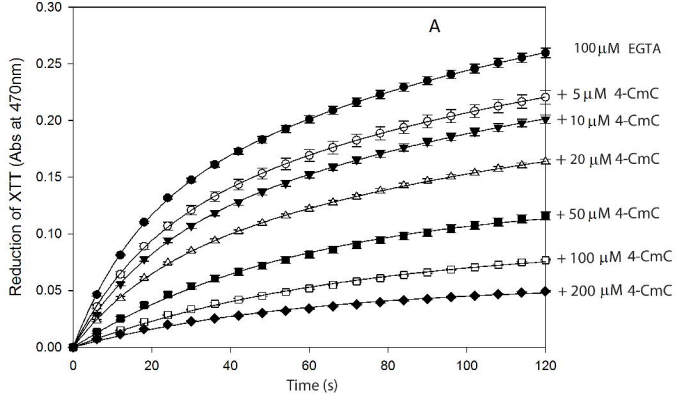


Figure 1


A
Reduction of XTT (Abs at 470nm)
Time (s)
100 µM EGTA
+ 5 µM 4-CmC
+ 10 µM 4-CmC
+ 20 µM 4-CmC
+ 50 µM 4-CmC
+ 100 µM 4-CmC
+ 200 µM 4-CmC

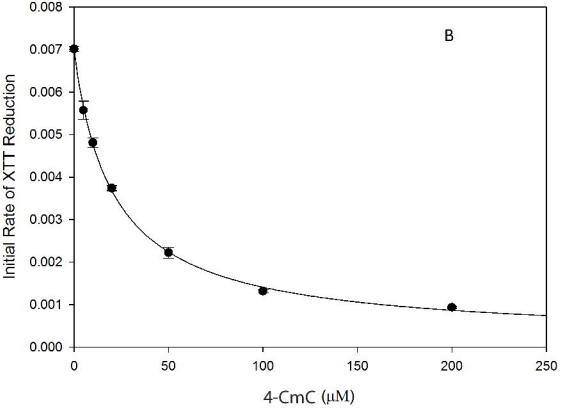

B
Initial Rate of XTT Reduction
4-CmC (µM)

Figure 2

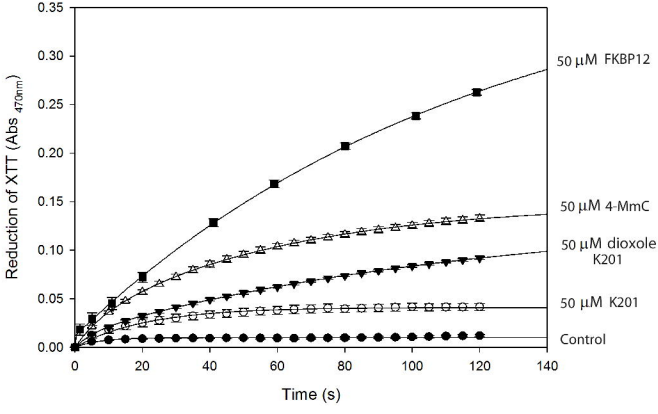
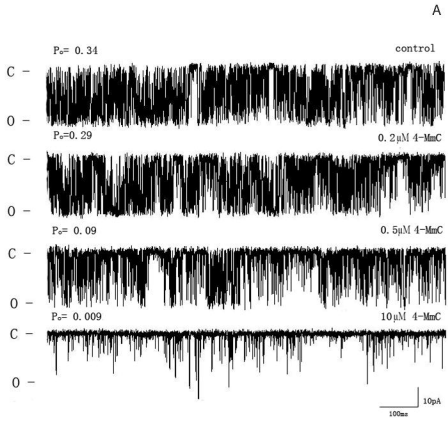
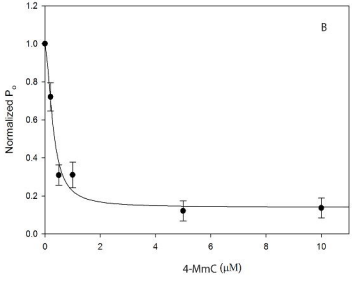

Reduction of XTT (Abs 470nm)
Time (s)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
20
40
60
80
100
120
140
50 μM FKBP12
50 μM 4-MmC
50 μM dioxole K201
50 μM K201
Control

Figure 3


A
Po= 0.34
control
C
O
Po=0.29
0.2μM 4-MmC
Po= 0.09
0.5μM 4-MmC
Po= 0.009
10μM 4-MmC
10pA
100ms


B
Normalized Po
4-MmC (μM)

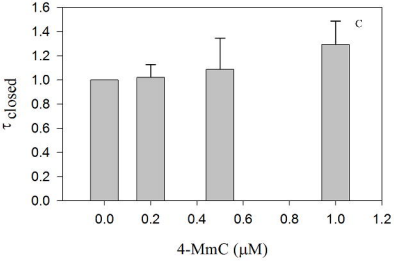
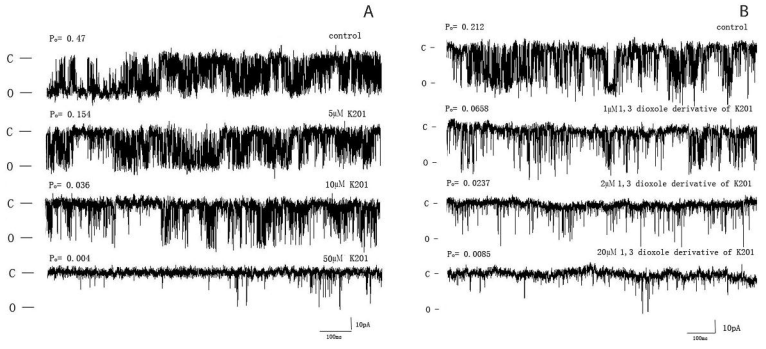
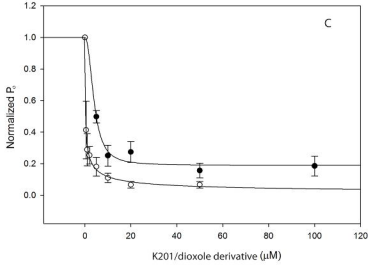
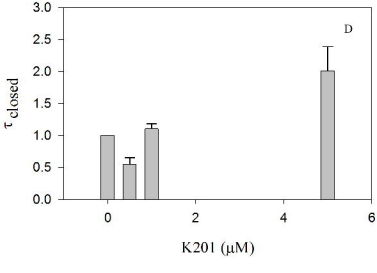

C
τ closed
4-MmC (μM)

Figure 4


A
B
control
5μM K201
10μM K201
50μM K201
1μM 1,3 dioxole derivative of K201
2μM 1,3 dioxole derivative of K201
20μM 1,3 dioxole derivative of K201
10pA
100ms


C
Normalized P2
K201/dioxole derivative (μM)


D
τ closed
K201 (μM)

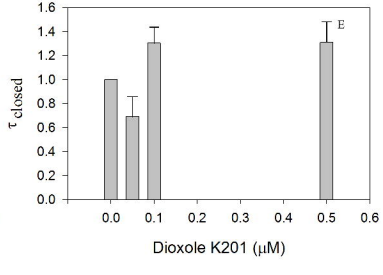

E
τ closed
Dioxole K201 (μM)

Figure 5

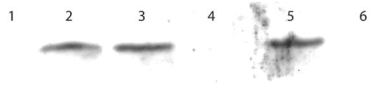
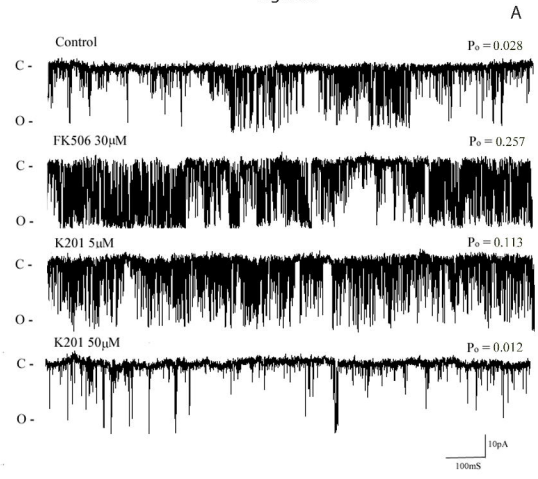


Figure 6


A
Control
Po = 0.028
C -
O -
FK506 30μM
Po = 0.257
C -
O -
K201 5μM
Po = 0.113
C -
O -
K201 50μM
Po = 0.012
C -
O -
10pA
100mS

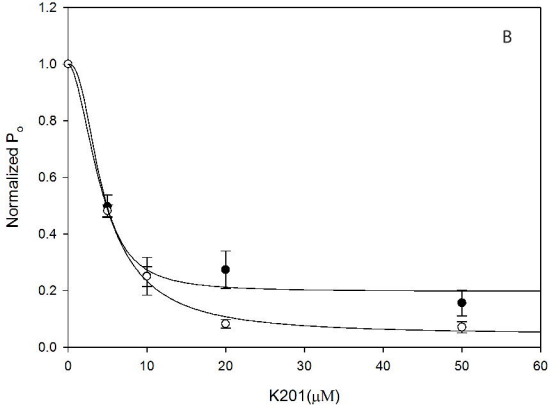
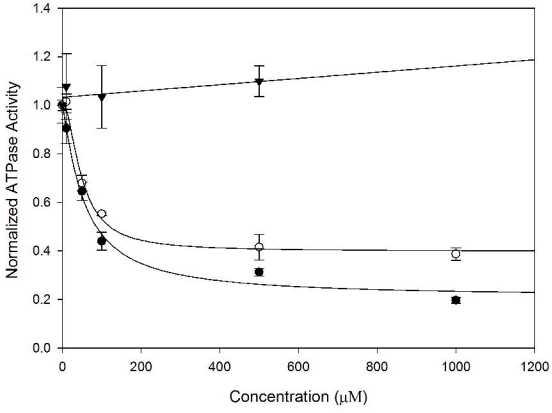

B
Normalized Po
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
K201(μM)

Figure 7


Normalized ATPase Activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
Concentration (μM)