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Potential role of cardiac calsequestrin in the lethal arrhythmic effects of cocaine*

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Abstract

Background—Cocaine-related deaths are continuously rising and its overdose is often associated with lethal cardiotoxic effects.

Methods and Results—Our approach, employing isothermal titration calorimetry (ITC) and light scattering in parallel, has confirmed the significant affinity of human cardiac calsequestrin (CASQ2) for cocaine. Calsequestrin (CASQ) is a major Ca^{2+}-storage protein within the sarcoplasmic reticulum (SR) of both cardiac and skeletal muscles. CASQ acts as a Ca^{2+} buffer and Ca^{2+}-channel regulator through its unique Ca^{2+}-dependent oligomerization. Equilibrium dialysis and atomic absorption spectroscopy experiments illustrated the perturbational effect of cocaine on CASQ2 polymerization, resulting in substantial reduction of its Ca^{2+}-binding capacity. We also confirmed the accumulation of cocaine in rat heart tissue and the substantial effects cocaine has on cultured C2C12 cells. The same experiments were performed with methamphetamine as a control, which displayed neither affinity for CASQ2 nor any significant effects on its function. Since cocaine did not have any direct effect on the Ca^{2+}-release channel judging from our single channel recordings, these studies provide new insights into how cocaine may interfere with the normal E-C coupling mechanism with lethal arrhythmogenic consequences.

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Conclusion—We propose that cocaine accumulates in SR through its affinity for CASQ2 and affects both SR Ca^{2+} storage and release by altering the normal CASQ2 Ca^{2+}-dependent polymerization. By this mechanism, cocaine use could produce serious cardiac problems, especially in people who have genetically-impaired CASQ2, defects in other E-C coupling components, or compromised cocaine metabolism and clearance.

Keywords

CASQ: calsequestrin; SR: sarcoplasmic reticulum; ITC: isothermal titration calorimetry; K_d: dissociation constant; RYR: ryanodine receptor; CPVT: catecholamine-induced polymorphic ventricular tachycardia

1. INTRODUCTION

Cocaine abuse continues to be a serious problem all over the world (Calcaterra and Binswanger, 2013) and the global number of annual cocaine users in 2010 ranged from 13.3 million to 19.7 million (Dargan and Wood, 2012). In the US alone, there were 1.4 million cocaine users in 2011 with as much as 19% of the US population admitting usage (Dargan and Wood, 2012). Unfortunately, in addition to numerous complications in heart, liver, kidney, and CNS (Valente et al., 2012), cocaine is the most frequent cause of drug-related deaths and emergency room visits in the U.S. (DAWN report 2010; National Institute on Drug Abuse (NIDA), 2002; Glauser and Queen, 2007). Since the re-emergence of recreational cocaine use in the late 1980s and recent appearance of an inexpensive freebase cocaine, illicit usage of cocaine has been linked to a dramatic rise in both acute and chronic cardiotoxicity (Phillips et al., 2009).

While cardiotoxic manifestations resemble traditional cardiac conditions symptomatically, the exact mechanism of cocaine-induced cardiac death remains unknown and thus unpredictable (Turillazzi et al., 2012; Basso et al., 2011; Maraj et al., 2010; Bauman et al., 1994), leaving the need for a better understanding of both cocaine-related cardiac complications and sudden cardiac death. Although myocardial infarction (MI) is considered the best documented complication of cocaine abuse, little evidence of MI was found during autopsies in which patients died suddenly after cocaine ingestion (Basso et al., 2011; Bauman et al., 1994). Instead, the cocaine-related cardiac death was most often arrhythmogenic in nature (Basso et al., 2011; Bauman et al., 1994). Likewise, significant prolongation of the QTc interval among cocaine users has been reported (Levin et al., 2008; Taylor et al., 2004; Garmouras et al., 2000). The QTc interval prolongation has been associated with catecholamine-induced polymorphic ventricular tachycardia (CPVT)-related sudden death. CPVT is due to mutations in either the cardiac calsequestrin (CASQ2), or in the cardiac Ca^{2+} release channel, ryanodine receptor (RyR2; de la Fuente et al., 2008; Lahat et al., 2001; Laitinen et al., 2001; Priori et al., 2001; Qin et al., 2008; Swan et al., 1999; Terentyev et al., 2008).

CASQ is a high capacity and low-affinity Ca^{2+} buffer protein in sarcoplasmic reticulum (SR) of skeletal muscle (CASQ1) and cardiac muscle (CASQ) and is highly conserved among vertebrates with a high level of sequence similarity (Sanchez et al., 2012; Wang et al., 1998). Human CASQ2 is known to have significant affinity for a number of

pharmaceutical drugs that have cardiotoxic side effects (Subramanian et al., 2012; Kang et al., 2010; Kim et al., 2007; Park et al., 2003; Wang et al., 1998). We have previously shown that accumulation of CASQ2-affinity compounds, such as tricyclic antidepressants, in the SR produces a continuous Ca^{2+} leakage through RyR probably through altering its Ca^{2+}-dependent polymerization and substantially reduces the SR Ca^{2+} (Kim et al., 2007). Many tricyclic antidepressants, as well as selective serotonin re-uptake inhibitors (SSRIs), have been connected to cardiotoxicity, and overdoses of some can result in fatal arrhythmia (Tarabar et al., 2008; Andrews and Nemeroff, 1994; Zima et al., 2008).

Overall, the physiological manifestation of cardiotoxicity from cocaine use is similar to the pathophysiology produced by CASQ2 mutations such as CPVT2. The physiological implication of this similarity is unclear so far and led us to investigate the molecular interactions between CASQ2 and cocaine and subsequent consequences. Drug association to CASQ2 usually results in a significant disruption of its Ca^{2+}-binding capacity, warranting a systematic study to investigate any connection between it and cocaine-related arrhythmia. In this report, another illicit substance, methamphetamine, was compared throughout the experiments as a negative control. Contrary to the serious cardioxicity of cocaine, the lethal effect of methamphetamine has been understood mainly through its neurotoxic effect to the central nervous system (Calcaterra et al., 2013).

2. MATERIALS AND METHODS

2.1. Isothermal titration calorimetry (ITC)

We used ITC to measure the differential binding affinities between CASQ2 and cocaine, CASQ2 and methamphetamine. For calorimetric measurements, purified CASQ2 was dialyzed for 3 days at 4 °C in 300 mM KCl and 10 mM MOPS, pH 7.5. 50 μM CASQ2 was titrated with a 2 mM cocaine or methamphetamine stock solutions made in the same buffer. The experiment consisted of 29 injections of 10 μL drug solution at 300-second intervals into the CASQ2 solution stirred at 300 RPM. Thermodynamic results were obtained by fitting the data to an *n*-sites equivalent binding model using nonlinear least-squares regression in Origin (OriginLab). The heat of binding was measured three times and averaged.

2.2. Molecular mass determination by multi-angle light scattering

The perturbation effect of cocaine on oligomeric status of CASQ2 was investigated in solution by a multi-angle laser light scattering. Chromatography and light scattering experiments were performed as previously described (Sanchez et al., 2011). For samples containing cocaine, 2 mg/mL CASQ2 was incubated with 350 μM cocaine and the same cocaine concentration was kept constant in the running buffer.

2.3. Equilibrium dialysis and atomic absorption spectroscopy

In order to estimate the cocaine effect on the Ca^{2+} fractional occupancy (Y = [bound Ca^{2+}] / [CASQ2]) for CASQ2, equilibrium dialysis and atomic absorption spectroscopy were performed as described previously (Sanchez et al., 2011). To investigate the effect of cocaine and methamphetamine on CASQ2 Ca^{2+}-binding capacity, 1 mg/mL CASQ2 was

incubated with 350 μM cocaine or methamphetamine for 1 hour prior to equilibrium dialysis.

2.4. Acute cocaine exposure and quantification of cocaine from cardiac tissue

A total of 10 Sprague-Dawley rats (Simonsen Laboratories.) weighing 300–400 g were housed as described (Browning et al., 2011). Care of the animals was done in accordance with the Guide for the Care and Use of Laboratory Animals and all procedures were approved by the Institutional Animal Care and Use Committee of Washington State University. To measure the effect of chronic cocaine, rats received a single intraperitoneal (IP) injection of 20 mg cocaine/kg body mass daily for up to 4 days. To track possible daily accumulation, animals (n = 2) were sacrificed 40 minutes post IP injection on days 1 through 4. For the control, two animals were injected daily with a single intraperitoneal injection of saline (1 mL/kg body mass) for 4 days and were sacrificed on day 4, 40 minutes after injection.

Cardiac muscle tissue was then removed, washed with ice cold PBS, pH 7.4 to remove excess blood, flash frozen in liquid nitrogen, and stored at −80 °C until needed. In order to prepare samples for analysis, cardiac tissue of varying weight and containing atrium, left and right ventricles was resuspended in 5 mL ice-cold PBS, pH 7.4, and washed five times. PBS was then removed and the tissue was resuspended in 5 mL ice cold 100 mM sodium carbonate containing 1 mM EDTA at pH 11.5. Homogenization was carried out by microsonication pulsation for 5 seconds, then 15 seconds on ice for 5 complete cycles. After sonication, the bicarbonate base of cocaine was extracted by addition of 10 mL diethyl ether, after thorough mixing, the organic phase was removed, dried to completion, and resolubilized in 1 mL of LC-MS/MS running buffer (0.05 % formic acid, 0.2 % acetic acid) containing 2.5 μM L-phenacitin (Sigma) as an internal standard.

Samples were analyzed using an 1100 series HPLC (Agilent Technologies) and an API 4000 tandem mass-spectrometry (Applied Biosystems/MDS Sciex). Chromatography was performed on a Synergi Polar reverse-phase column (30 × 3.0 mm, 4-μm; Phenomenex) using 0.05 % formic acid and 0.2 % acetic acid in water for phase A and 9.9 % water and 0.1 % formic acid in CH_3CN for phase B. The column was equilibrated at initial conditions of 95 % mobile phase A for 0.3 minutes. Chromatographic separation was achieved using a linear gradient over the next 2.2 minutes to 25 % phase A. Mobile phase A was then held at 25 % over 0.5 min, followed by a linear gradient back to 95 % A over 0.5 minutes. The column was re-equilibrated to the initial conditions over the next 1.5 minutes. The total chromatographic assay time was 5 minutes per sample. The retention times for internal standard and metabolite were 2.01 and 2.17 minutes, respectively. The optimized cocaine mass spectrometer parameters were set as previously described (Barr and Jones, 2011). Cocaine and the L-phenacetin internal standard were detected using positive ion multiple reaction-monitoring mode by monitoring the *m/z* transition from 304.4 to 149.9 (Supplement Figure 1) and 180 to 64.9, respectively. The product was quantified by extrapolation using a 0 to 10 μM cocaine standard curve. A chromatogram for internal standard and metabolite is shown in Supplemental Figure 1.[1]

2.5. Molecular docking

The structures of CASQ2 (PDB ID: 2VAF) and cocaine (NCBI PubChem CID 5760) were converted into PDBQT format by AutoDock Tools for usage in AutoDock 4.2 (Morris et al., 2009). A blind docking approach was performed enclosing the whole protein inside a large grid box. The atomic affinity grids were calculated using the AutoGrid module. All search parameters were kept default, except for the torsional rotation, which was reduced from 50° to 25° in order to increase the exhaustiveness of search. A total of 70 cocaine-docking runs were completed using the Lamarckian Genetic Algorithm output, resulting in the 70 best bound-conformations. The predicted binding orientations within each cluster were ranked hierarchically in order of increasing binding free energy (ΔG_b) via cluster analysis with a 2 Å RMSD cut-off, whereby the conformations within each cluster were ranked hierarchically in the order of increasing ΔG_b, the free energy of binding.

2.6. Single channel recordings

Artificial lipid bilayers contained a 5:4:1 mixture (50 mg/ml in decane) of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine. Bilayers were formed across a 100 μm hole. After a stable bilayer was formed, single rat RyR2 channels were incorporated following established methods (Qin et al., 2009). The luminal solution contained 10 mM CaHEPES (pH 7.4). Single RyR2s were exposed to this high luminal Ca^{2+} level for over 10 minutes so endogenous CASQ2 attached to the RyR2 has likely dissociated (Qin et al., 2009). No CASQ was added and thus single RyR2 examined were likely CASQ-free. The cytosolic solution contained 120 mM TrisHEPES (pH 7.4), 1 mM free Mg, 5 mM total ATP, 1 mM EGTA and 20 μM free Ca^{2+}. The required buffer mixture was calculated using the WinMAXC 2.05 program (Stanford University). Data acquisition and analysis was done using pClamp software (Axon CNS Molecular Devices). Single RyR channel recordings were sampled at 20 kHz and filtered at 1 kHz. Open and closed dwell times were defined from idealized recordings.

2.7. Ca^{2+} release from C2C12 cells

C2C12 (ATCC) mouse myoblast cells were seeded into 100 mm plates at a cell density of 1×10^6 and were maintained with DMEM containing 10 % FBS and 1 % penicillin / streptomycin at 37 °C in 5 % CO_2. Cells were maintained below 50 – 60 % confluence through regular passaging. Differentiation of C2C12 myoblasts was achieved by seeding 45 mm cell plates with approximately 5×10^5 cells. Cells were then grown to 100 % confluence and the media was changed to Dulbecco's Modified Eagle Medium (DMEM) containing 2 % horse serum with 1 % penicillin / streptomycin. Differentiation medium was replaced daily for eight days, after which cells were prepared for microscopy by rinsing them with Krebs-Ringer-HEPES (KRH) buffer and then treating them for 4 hours with a final concentration of 175 μM cocaine or methamphetamine. The cells were then treated for 4 hours with media containing analyte. After drug treatment, the cells were rinsed with KRH buffer and incubated with 1 μM Fluo-4AM (Invitrogen) for 30 minutes. Excess dye was removed by washing cells again with buffer A. Cells were then placed into a custom-built

[1]Supplementary material can be found by accessing the online version of this paper at http://dx.doi.org and by entering doi:...

incubation chamber mounted on an Axiovert 200M fluorescence microscope equipped with a 10X Achroplan 0.25NA objective lens, a Fluoarc HBO-100 mercury arc lamp and an ORCA-ER cooled charge-coupled device (CCD) (Hamamatsu). Chamber temperature was maintained at 37 °C using an Air-Therm airstream incubator. Fluorescence excitation was accomplished using a 470/40 nm band-pass filter and fluorescence emission monitored using a 525/50 nm band-pass filter. Images were obtained with 2×2 binning for a total of 4 minutes at 2.5-second intervals using Axiovision software (Carl Zeiss, Inc.). 10 mM caffeine was added to a final concentration of 2.5 mM in order to induce Ca^{2+} release. Data was analyzed using ImageJ (NIH) software by importing each dataset image stack and selecting nine random regions in each. Datasets were then background corrected and the values for the intensity of each region were plotted as a function of F/F_o. The number of independent events, as defined by a signal above $F/F_o = 1$, was then averaged for each of the nine regions of interest to determine the average number of Ca^{2+} waves/oscillation for each dataset (Lorenzon et al., 1997).

3. RESULTS

3.1. Affinity of cocaine for CASQ2

The binding affinity of cocaine for CASQ2 was determined using isothermal titration calorimetry (ITC). Results show CASQ2 binds cocaine with a K_d of 63.5 ± 2.7 μM and displays no significant affinity for methamphetamine (Fig. 1).

3.2. Monitoring the effect of cocaine binding on CASQ2 polymerization

To determine the effect of cocaine on Ca^{2+}-induced CASQ2 oligomerization, the molecular weight of CASQ2 in the presence and absence of cocaine was compared by the multi-angle light scattering (MALS). In the absence of Ca^{2+}, CASQ2 molecules were approximately 75% monomeric ad 25% dimeric (Fig. 2A, solid line). In the presence of 1 mM Ca^{2+}, CASQ2 monomers transitioned to a predominantly dimeric state (Fig. 2A, dotted line). However, addition of 350 μM cocaine to the Ca^{2+}-free solution increased the CASQ2 dimer population with some tetramers or higher ordered polymers observed (Fig. 2B, solid line). In the solution containing both 350 μM cocaine and 1 mM Ca^{2+}, the cocaine strongly perturbed the Ca^{2+}-dependent oligomerization of CASQ2 as shown by its broadened elution profile (Fig. 2B, dotted line).

3.3. Atomic absorption spectroscopy

Equilibrium dialysis followed by atomic absorption spectroscopy was used to determine the effect of cocaine on CASQ2 Ca^{2+}-binding capacity. In the presence of 350 μM cocaine (filled circle), CASQ2 reaches a maximal Ca^{2+} capacity of approximately 10 mol Ca^{2+} / mol CASQ (Fig. 3). When compared with CASQ2 with no cocaine (open triangle), there was approximately 50% reduction in the Ca^{2+} binding capacity due to a presence of 350 μM cocaine. However, 350 μM methamphetamine (filled rectangle), which showed no significant affinity for CASQ2 (Fig. 1), had no affect on CASQ2 Ca^{2+}-binding capacity (Fig. 3).

3.4. Cocaine accumulation within cardiac tissue

Following previously-established LC-MS/MS techniques for quantifying cocaine (Kronstrand et al., 2004), we determined cocaine concentrations above a 5 ng cocaine / g cardiac tissue threshold. After 40 minutes post-acute dosage, intact cocaine was present at concentrations ranging from 0.799 ± 0.051 μg /g for Day 1 on which animals were exposed to cocaine in acute doses, to 0.846 ± 0.070 μg /g for Day 2, 0.799 ± 0.069 μg/g for Day 3, and 0.795 ± 0.063 μg /g for Day 4 repeatedly-exposed animals (Fig. 4).

3.5. Molecular docking

Molecular docking predicted that 80 % of the bound conformations were found within the S1 site, a hydrophobic cavity with an inner narrow sub-cavity and larger outer sub-cavity formed between the three CASQ domains (Fig. 5). The calculated ΔG_b values for cocaine association to the S1 central cavity were from −7.83 to −7.31 kcal/mol and contained the most homogenous bound cocaine orientations (Fig. 5C). The remaining 20 % of the docked cocaine molecules were clustered in several other distinct pockets as illustrated by Figure 5A. S2-D1 and S2 were the pockets offered by the thioredoxin fold of Domain I and Domain III (Fig. 5D). S3-DII/DI and S3-DII/DIII are the cavities of the Domain I and Domain II inter-domain space and the Domain II and Domain III inter-domain space, respectively (Fig. 5E).

3.6. Cocaine effect on single channel

The action of cocaine on single RyR2 channel function was tested. Fig. 6A shows sample single channel recordings before and after addition of 500 μM cocaine. Fig. 6B shows representative all-points histograms before and after the cocaine application. The current difference between closed (0 pA) and open peaks was similar with or without the drug. The relative areas of the closed and open peaks (Po) with or without the drug were also similar. Control Po and current amplitude were 0.73±0.05 (n = 18) and 1.5±0.1 pA (n = 18), respectively. After the cocaine application, the Po and current amplitude were 0.71±0.10 (n = 3) and 1.4±0.3 (n = 3). These values are not significantly different than the control values. Fig. 6C shows the distributions of open and closed times with or without the drug. Cocaine had no detectable action on single RyR2 dwell times. In absence of cocaine, the arithmetic mean open time (MOT) and mean closed time (MCT) were 12.6±5 ms and 3.0±0.6 ms, respectively. After cocaine application, the MOT and MCT were 11.1±9 ms and 2.8±1.3 ms, respectively.

3.7. Ca^{2+} release from the C2C12 cell line

Cultured C2C12 mouse myoblasts were used to determine the effect of cocaine on Ca^{2+}storage and release. Individual images were then re-stacked, and random regions of interest were selected and background subtracted intensity was averaged and plotted (Fig. 7). After exposure to either methamphetamine or DMSO-vehicle, C2C12 exhibited robust transient Ca^{2+} release following caffeine addition. As shown in the representative traces in Fig. 7A, after the initial increase in fluorescence due to changes in osmotic pressure, both the DMSO-vehicle and methamphetamine exhibit Ca^{2+} waves for approximately 75 - 90 seconds. The cells pretreated with cocaine displayed a drastic reduction and in some cases,

showed complete ablation of Ca^{2+} waves (Miller et al., 2005). Figure 7a shows the representative traces for a single region of the control, cocaine, and methamphetamine treated cultures. The averaged frequency of Ca^{2+} waves, which is a count only for which $F/F_o > 1$ in each region, are presented in Fig. 7b. Overall, the frequency of Ca^{2+} waves in cocaine-treated samples was reduced more than one order of magnitude compared to either the control or methamphetamine-treated cells.

4. DISCUSSION

To understand the molecular mechanism of lethal cardiotoxicity of cocaine, we examined its effect on two critical components of cardiac SR, CASQ2 and Ca^{2+} release channel. Our results clearly showed that cocaine has a significant affinity to CASQ2 and exhibit an inhibitory effect on both Ca^{2+}-dependent polymerization and Ca^{2+}-binding capacity of CASQ2. We also confirmed the accumulation of cocaine in rat heart tissue and its substantial effects on cultured C2C12 cells. Human, mouse, and rat CASQ2 invested in this work have 86 % sequence identity (Sanchez et al., 2012). Considering the fact that the majority of these differences are in the relatively disordered C-terminal tail region and that the number and location of known post-translational modification sites are conserved Sanchez et al., 2011; Sanchez et al., 2012), all three CASQ2s likely have the same global structure and consequently, to behave similarly, with respect to Ca^{2+}-buffering capacities, calcium-dependent polymerization, and protein-drug interactions.

A transient elevation in intracellular Ca^{2+} concentrations resulting from SR Ca^{2+}-release produces muscular contraction, while active transport of Ca^{2+} by Ca^{2+}-ATPases back into the SR produces relaxation (MacLennan, 2000; Mudd and Kass, 2008). Serious physiological problems result from even slight perturbation of this tightly- regulated cardiac SR process (Berchtold et al., 2000; Dulhunty, 2006; Gergs et al., 2007; Györke and Terentyev, 2008; Knollmann and Roden, 2008; MacLennan, 2000; Yuan et al., 2007). One of the primary SR proteins contributing to this excitation-contraction coupling event is the Ca^{2+}-storage/buffer protein, CASQ. CASQ is located within both cardiac muscle and skeletal muscle, CASQ2 and CASQ1 respectively. CASQ binds Ca^{2+} with moderate affinity but very high capacity over the physiological Ca^{2+} concentration range and releases it at a high rate (MacLennan and Wong, 1971; Park et al., 2004). CASQ also facilitates further uptake of cytosolic Ca^{2+} by Ca^{2+}-ATPases by acting as a Ca^{2+} buffer inside the SR, lowering free Ca^{2+} concentrations while simultaneously localizing Ca^{2+} at the Ca^{2+} release site in the SR lumen (Chen et al., 1996; Launikonis et al., 2005). CASQ regulates the amount of Ca^{2+} released through the ryanodine receptor (RyR) (Gyorke et al., 2004; Paolini et al., 2007; Terentyev et al., 2003) while also serving as a luminal Ca^{2+} sensor for RyR (Terentyev et al., 2003; Tijskens et al., 2003; Wang et al., 1998; Yano and Zarain-Herzberg, 1994).

Abnormal CASQ2 function is the cause of many pathologic manifestations including catecholamine-induced polymorphic ventricular tachycardia (CPVT2), an arrhythmogenic disorder characterized by syncope and sudden cardiac death (Eldar et al., 2003; Knollmann and Roden., 2008; Kontula et al., 2005; Postma et al., 2002; Terentyev et al., 2008) to which specific CASQ2 mutations have been linked. CASQ2 displays significant binding affinity

for several classes of drugs associated with cardiotoxic side-effects (Kang et al., 2010). Indeed, the interaction of these agents with CASQ2 likely contributes to their cardiotoxic effects.

Our ITC and LC-MS/MS results showed a significant affinity of CASQ2 for cocaine, 63.5 μM (Fig. 1) and existence of ~0.8 μg cocaine per gram of heart tissue (Fig. 4). Cocaine concentrations were determined using a base-extraction procedure (pH 11.5). This basic condition, known to cause non-enzymatic cocaine hydrolysis, was not controlled for by inclusion of an internal standard subject to similar conditions; therefore, actual tissue cocaine concentrations may be slightly higher than reported here. However, we did not observe a time-dependent increase of intact cocaine molecules in the repeatedly-exposed rats, which is very likely due to an *in vivo* conversion of injected cocaine into primary or secondary metabolites that could easily escape our detection method. The concentration of cocaine and its metabolite could be much higher if cardiac SR were enriched and monitored as we have done for other cardiotoxic compounds (Kim et al., 2005).

We also explored cocaine action to the high-capacity Ca^{2+} binding by CASQ, which is directly linked to its Ca^{2+}-dependent polymerization (Cho et al., 2007; Gatti et al., 2001; Park et al., 2004, 2003). Ca^{2+}-binding by CASQ involves Ca^{2+} largely filling the negatively-charged dimer interface, cross-bridging them and eventually forming a linear polymer. Our MALS data clearly indicates that cocaine-binding significantly affects CASQ2 Ca^{2+}-induced polymerization, inducing Ca^{2+}-independent premature CASQ2 oligomerization (Fig. 2). The reduced CASQ2 Ca^{2+}-binding capacity due to presence of cocaine (Fig. 3) is also consistent with our demonstration that cocaine-exposed C2C12 cells displayed substantially decreased caffeine-induced Ca^{2+} release (Fig. 7).

Tsushima et al (1996) tested cocaine action (1 to 10 mM) on single RyR2 channels. They reported that it acts as a RyR2 open channel blocker with an apparent dissociation constant of 38 mM at 0 mV. Open channel blocking implies the cocaine rapidly enters and exits the RyR2 open pore, generating a voltage-dependent fast flicker blockade. Their CHAPS-solubilized dog single RyR2 channel data were collected in symmetrical 250 mM KCl (no cytosolic Mg^{2+} or ATP, no added luminal Ca^{2+}). Our single channel results indicated there was no detectable action of cocaine at 500 μM on native rat single RyR2 channels. These apparently contradictory results are likely explained by the very different recording conditions and cocaine concentrations used. Note that our studies were done in recording conditions designed to better reflect a cellular reality (0 mV, cytosolic MgATP, luminal Ca^{2+} present). Tsushima et al (1996) understandably used the recording conditions that would optimize the current signal-to-noise ratio (large potential, large concentration of a monovalent charge carrier, absence of luminal Ca^{2+}). Our lower signal-to-noise and relatively small current at 0 mV may have precluded detection of a subtle fast flicker block. It is also important to note that the cocaine concentration in cardiac cells (in patients) very likely never exceeds 1 mM (Rees et al., 2013 Bystrowska et al., 2012; Giroud et al., 2004; Patel, 1996; Peretti et al., 1990). In addition, the SR membrane potential is not likely to stray far from 0 mV (Somlyo et al., 1985). Thus, cocaine may indeed act as a RyR2 flicker blocker at very high, non-physiological concentrations, but it does not at the cocaine levels

likely to exist in working hearts. Here, we showed that 500 μM has no detectable action on single RyR channel function.

Analysis of our docking results (Fig. 5) indicated that cocaine mainly binds to CASQ2 by inserting its benzoyl moiety into the S1 site (Fig. 5A, 5C). The thioredoxin domains in CASQ2 also provided a binding pocket for cocaine, albeit with a lower affinity. The potential binding sites were located at the CASQ dimer interface and thus cocaine binding to any of those sites would significantly alter the intermolecular interface and subsequent Ca^{2}-dependent CASQ oligomerization. As shown in Fig. 5C, when bound, the ecgonine cocaine moiety binding can disrupt the R231-D244 salt bridge have been previously shown to be important for driving conformational changes in response to Ca^{2+} (Sanchez et al., 2012). The second potential impact of cocaine association was found at the site in the thioredoxin-like fold in domain III where insertion of the cocaine benzoyl moiety could disrupt the thioredoxin-like fold hydrophobic core, and considering the majority of unique bound conformers found in this particular environment, cocaine could provoke similar conformational changes seen in certain CPVT phenotypes. Another CASQ-cocaine interaction was the apparent hydrogen-bond formation between the cocaine o-methyl-ecgonine moiety and adjacent polypeptide amides in a potentially disruptive location within the domain-interface (Fig. 5E).

Binding of cocaine to CASQ2 reduces its Ca^{2+}-binding capabilities and Ca^{2+}-dependent polymerization, and thus could alter the SR Ca^{2+}-buffering capacity and response rate. We have previously shown that hydrophobic drugs with CASQ-affinity can accumulate in purified SR (Kim et al., 2007). Here we show that a significant amount of cocaine was detected in heart tissue of rats dosed with cocaine. Given the fact that CASQ is the most abundant protein (100–500 mg/mL) in heart tissue along with our evidence that CASQ2 contains several putative cocaine-binding pockets, it is tempting to speculate that the observed cardiac complications and lethality of cocaine are at least partially due to inducing CASQ2 functional abnormalities. One possibility is that the significantly reduced CASQ2 Ca^{2+}-binding capacity due to cocaine binding increases the free SR luminal Ca^{2+} concentration beyond the store-overload-induced Ca^{2+} release (SOICR) threshold (MacLennan and Chen, 2009). A slight alteration in normal cardiac SR physiological functions is enough to cause serious problems in most individuals. In the case of habitual cocaine abusers, its accumulation within the SR can easily generate cumulative, chronic toxicity. Individuals who have compromised CASQ2 due to genetic reasons such as CPVT2 may be at a higher risk for cocaine cardiotoxicity. For example, our previous data have shown that all CPVT-related CASQ2 mutations result in disrupted Ca^{2+}-binding capacity and Ca^{2+}-dependent polymerization (Kim et al., 2007), similar to the interference displayed by cocaine and other CASQ2-affinity compound drugs. Hereditary CASQ2 mutations alone may be benign enough not to cause arrhythmias, but upon exposure to cocaine and other aforementioned CASQ-affinity drugs, arrhythmias or other cardiac complications could manifest because of interference with normal CASQ2 functions due to an additive or synergistic between cocaine and hereditary mutations. Therefore, specific caution and education are required for people at a higher risk of serious cardiac complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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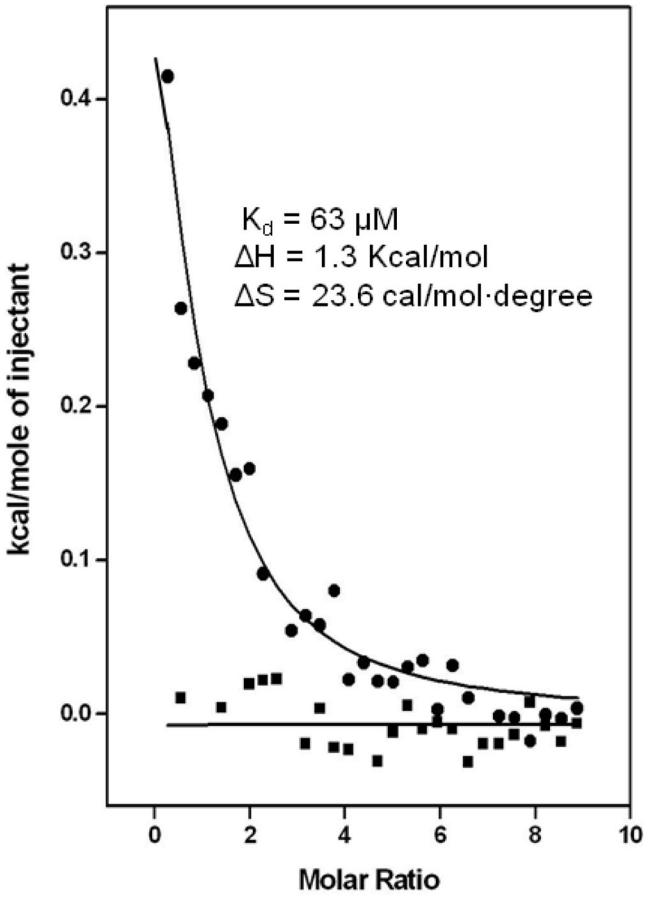

Fig. 1. Measurement of the heat released upon cocaine and methamphetamine injection by ITC

The trend of heat released by serial injections of ether cocaine (●) or methamphetamine (■). 50 μM CASQ2 was titrated with a 2 mM stock solution of cocaine or methamphetamine dissolved in ITC buffer (300 mM KCl, 10 mM MOPS, pH 7.5). The X-axis represents the molar ration of [Ligand] / [Protein]. The heat of binding was measured three times and averaged. The corresponding ΔH, ΔS and K_d values for cocain-binding are indicated.

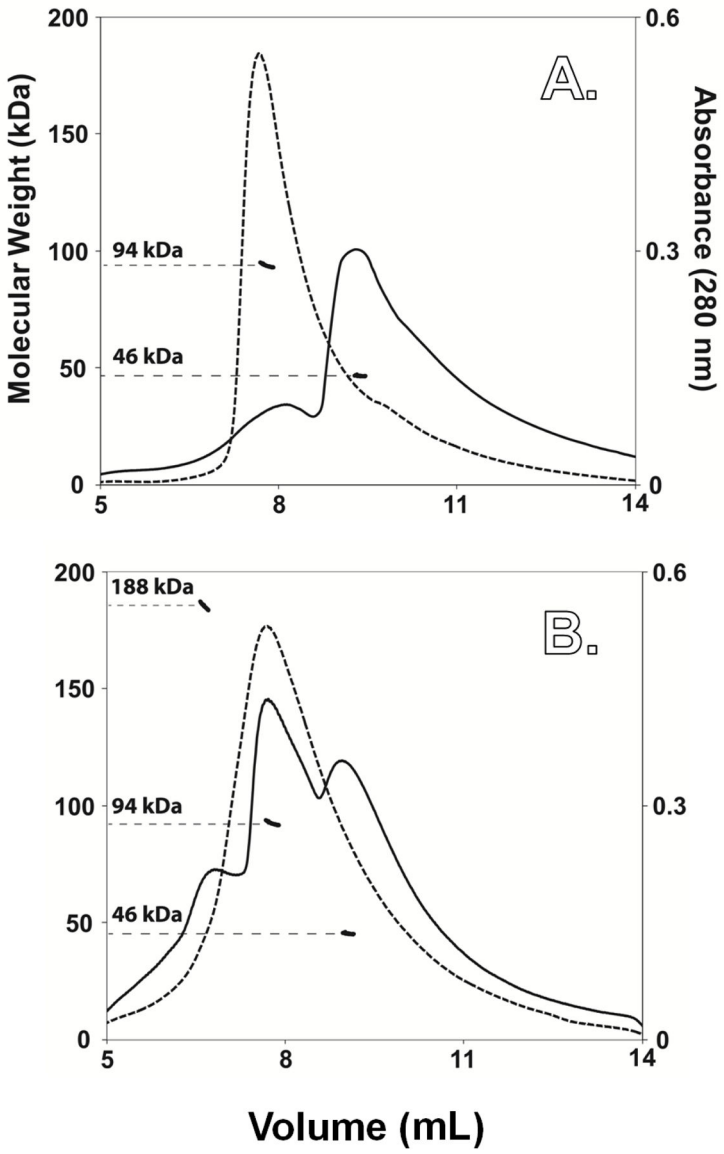

Fig. 2. CASQ2 oligomeric states with and without cocaine

Elution profiles were monitored by multi-angle laser light-scattering (left Y-axis) and 280 nm UV absorption (right Y-axis) versus elution volume (X-axis). The solid line indicates the UV absorption profile in the absence of $CaCl_2$, and the dotted line represents the UV absorption profile in the presence of 1 mM $CaCl_2$. **A)** The monomer to dimer transition of CASQ2 resulting from no Ca^{2+} (solid line) to 1 mM Ca^{2+} (dotted line) in absence of cocaine. **B)** The transitional pattern from no Ca^{2+} (solid line) to 1 mM Ca^{2+} (dotted line) of CASQ2 in the presence of 350 μM cocaine. The molecular weights of elutes as determined by multi-angle laser light scattering are shown as dots at the center of each peak.

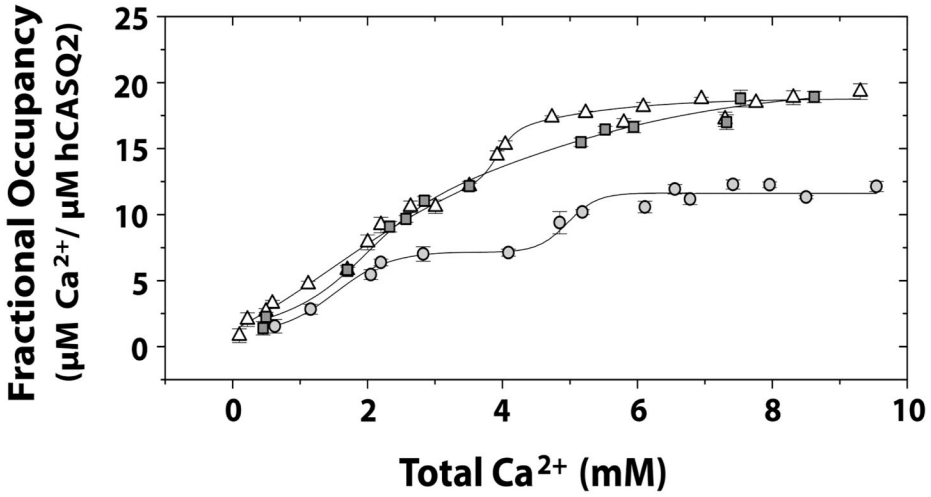

Fig. 3. CASQ2 Ca^{2+}-binding capacity inhibition by cocaine

The number of Ca^{2+} ions bound to CASQ2 was determined through equilibrium dialysis and atomic absorption spectroscopy. Fractional occupancy (y = [bound Ca^{2+}]/[total protein]) is plotted against [unbound Ca^{2+}] for CASQ2 without drugs (open triangle), in the presence of 350 μM cocaine (filled circle), and in the presence of 350 μM methamphetamine (filled rectangle).

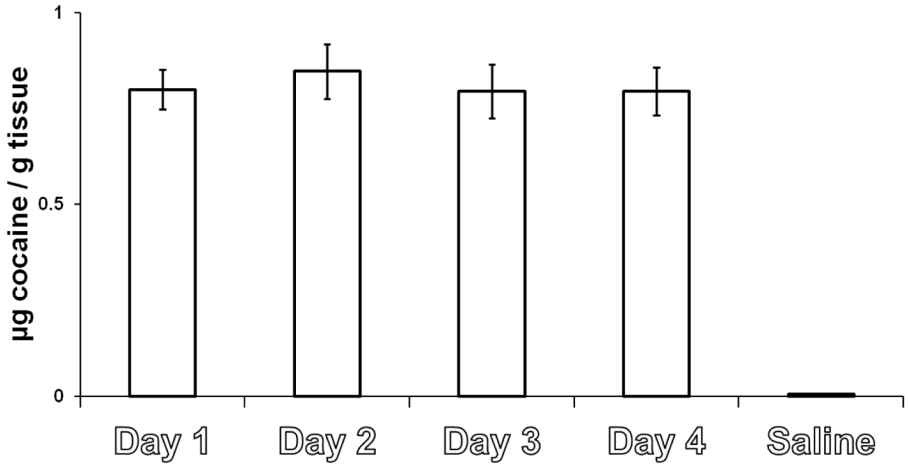

Fig. 4. Quantification of cocaine isolated from heart tissue

Relative quantification µg/mg of tissue for animals treated daily with 20 µg/g body weight of cocaine or saline treated samples. Cocaine concentrations were determined using a base-extraction procedure (pH 11.5). This basic condition, known to cause hydrolysis, was not controlled for by inclusion of an internal standard subject to similar conditions; therefore, actual tissue cocaine concentrations may be slightly higher than reported here.

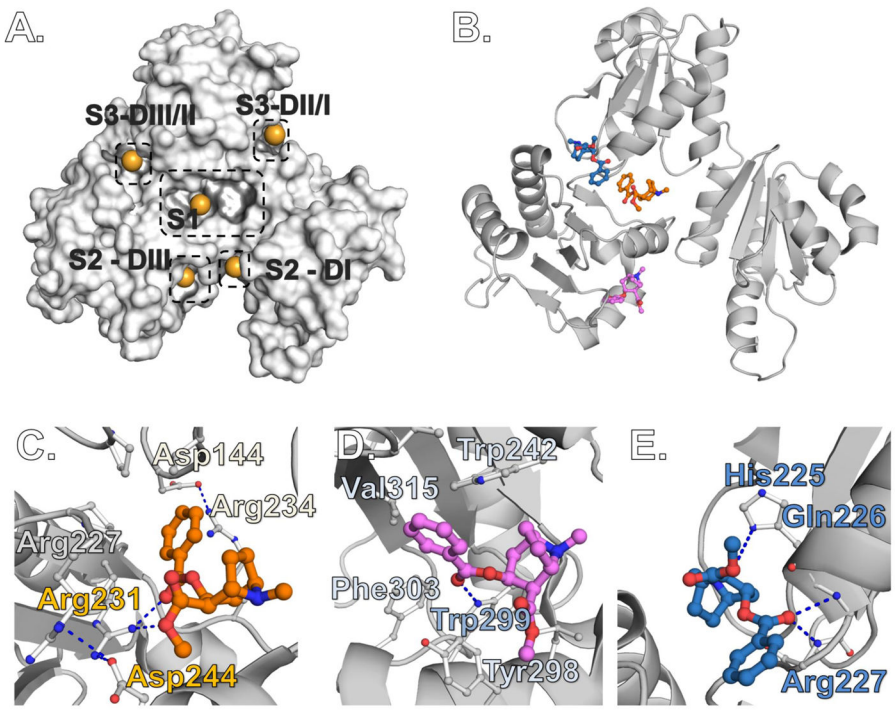

Fig. 5. Surface representation of the CASQ2-cocaine complex

A) All the cocaine-binding positions as determined by docking are represented as brown spheres. **B)** Ribbon diagram representing the three major cocaine-binding sites. The orange ball and stick represents S1 binding, green represents the S2 binding site, and purple represents S3 binding. **C)** A close up of the proposed S1 binding with residues and electrostatically-interacting residues shown in orange. **D)** Representation of S2 binding and the local binding environment. **E)** Representation of the S3 binding site with its constituent residues shown. These figures were generated using Open-Source software PyMOL™ (v1.4).

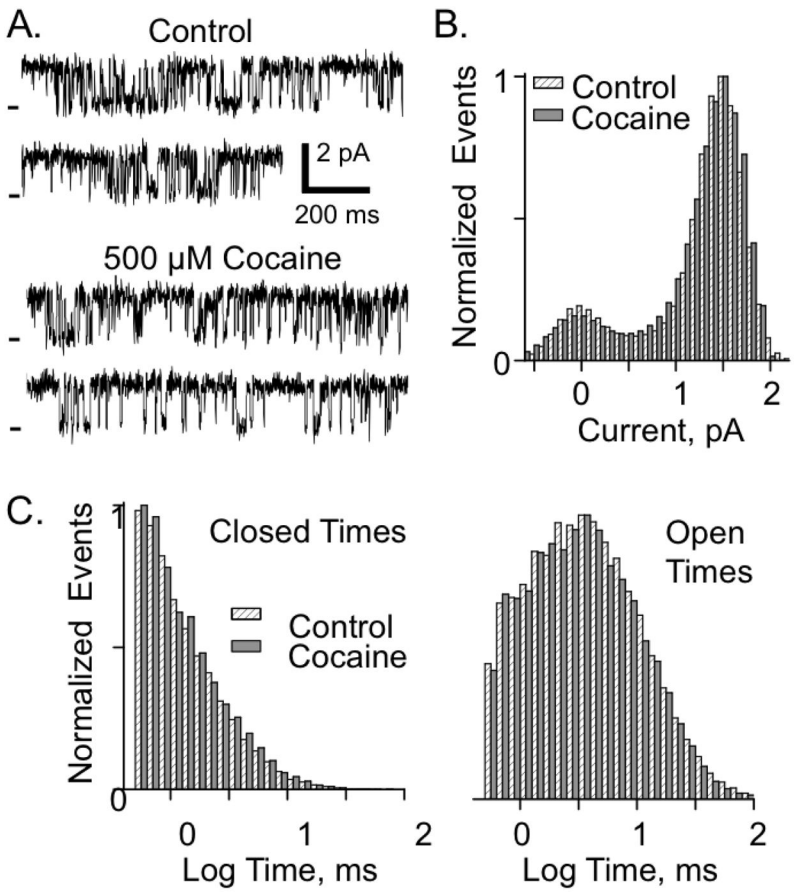

Fig. 6. Cocaine has no detectable action on RyR2 function

Endogenous CASQ was removed from the single RyR2 channels and no exogenous CASQ was added. **A)** Sample single channel recordings. Opening events are shown as upward deflections from the marked zero current level (left margin). Membrane potential was 0 mV. Cocaine was added to cytosolic solution. **B)** All-points histograms from 4-minute recordings in the presence and absence of 500 μM cocaine. **C)** Sample open and closed dwell time histograms in the absence or presence of 500 μM cocaine.

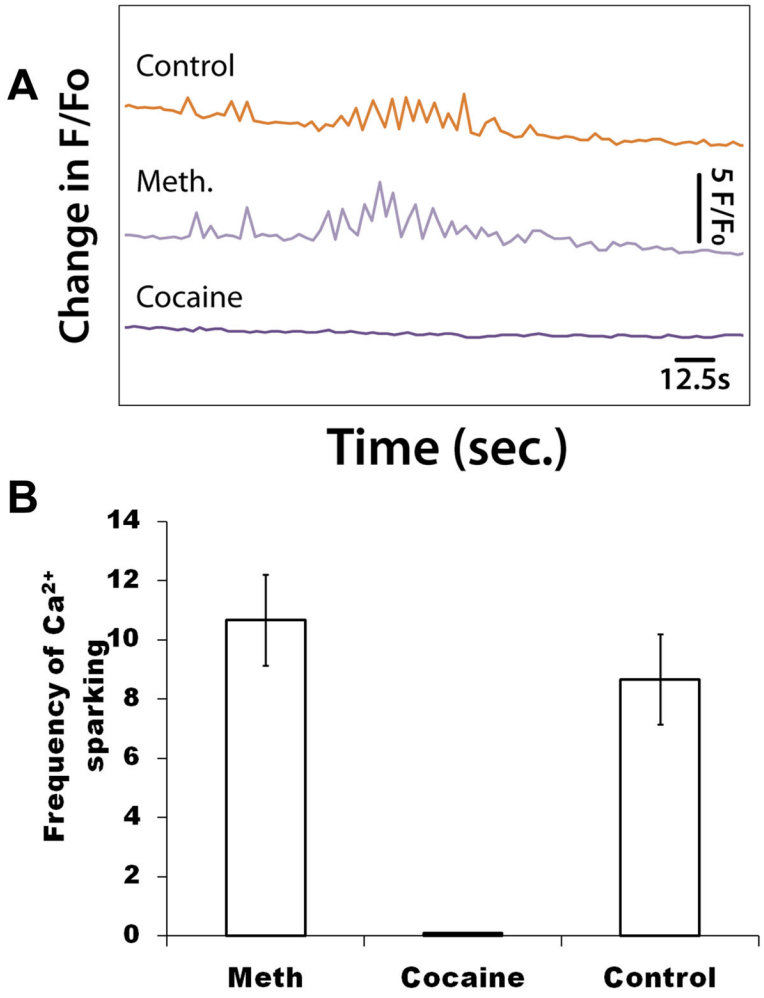

Fig. 7.

A) Traces of observed F/F_0 for C2C12 Myocytes incubated with DMSO vehicle (control), 175 μM methamphetamine, and 175 μM cocaine. **B)** Histogram representation showing the average Ca^{2+} waves for each dataset.