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Interaction between Cardiac Calsequestrin and Drugs with Known Cardiotoxicity

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ABSTRACT

Ca^{2+} regulation is coupled to critical signals in eucaryotic cells, and calsequestrin is one of the crucial components for this calcium regulation. Our previous observations of calsequestrins revealed the existence of three thioredoxin-like folds, a basic motif that often provides the platform for small molecule binding. Therefore, we have examined the previously reported trifluoperazine and other pharmaceuticals that have similar heart-related side effects (such as tachycardia; bradycardia; palpitation; changing PR, QRS, QTc intervals in electrocardiogram; heart failure) for their binding affinity to cardiac calsequestrin (cCSQ) using isothermal titration calorimetry. Our results showed that several antipsychotic phenothiazine derivatives, tricyclic antidepressants, and anthracycline derivatives bind cCSQ with K_d in the micromolar range. For these compounds that have a significantly low K_d, their effect on Ca^{2+} binding capacity of cCSQ was checked using equilibrium dialysis and atomic absorption spectroscopy, which clearly showed a significant reduction in Ca^{2+} binding capacity of cCSQ as a result of this interaction. Furthermore, 8-anilino-1-naphthalene sulfonate (ANS) binding to cCSQ closely resembles ANS binding to flavine or nucleotide binding sites. The combination of this information with the high abundance of CSQ in SR and the high membrane permeability of those drugs led us to the specific hypothesis that there are undesirable and damaging interactions between cCSQ and tricyclic antidepressants, phenothiazine derivatives, anthracyclines, and many other pharmaceutical compounds and to the corollary hypothesis that better understanding of the molecular details of cCSQ-drug interactions could lead to modified drug molecules with reduced heart-related side effects.

Calsequestrin (CSQ) is a major Ca^{2+} storage protein within the sarcoplasmic reticulum (SR) of both cardiac and skeletal muscle. Muscle CSQ has high capacity and low affinity for Ca^{2+}, binding about 40 to 80 ions per molecule with a binding constant of about 1 mM under physiological conditions (MacLennan and Wong, 1971; Park et al., 2004). Not only does CSQ act as a Ca^{2+} buffer inside the SR, lowering free Ca^{2+} concentrations and thereby facilitating further uptake by the Ca^{2+}-ATPases, but also CSQ actively participates in muscle contraction by localizing Ca^{2+} at the release site and regulating the amount of Ca^{2+} released through the ryanodine receptor (MacLennan et al., 2002). Therefore, CSQ has been thought to be essential for a short contraction/relaxation cycle that can be completed within a time interval of less than 100 ms (Mitchell et al., 1988; Collins et al., 1990; Damiani and Margreth, 1990; Yano and Zarain-Herzberg, 1994; Jones et al., 1995, 1998; Zhang et al., 1997; Ohkura et al., 1998; Kobayashi et al., 2000; Terentyev et al., 2003; Tijskens et al., 2003).

The overall structures of both cardiac and skeletal CSQs (cCSQ, sCSQ) are composed of three thioredoxin-like domains, even though no tandem repeats were evident in their primary sequences (Wang et al., 1998; Park et al., 2004). Individual thioredoxin-like domains have a five-stranded β-sheet sandwiched by four α-helices, composed of ~100 residues. Almost without exception, thioredoxin-like folds bind small molecular ligands or have a redox site at a specific locus (Branden, 1980; Katti et al., 1990). These sites occur at chain reversals that generate a crevice defined by the edge of one β-sheet and the carboxyl ends of the adjacent β2 and β3 strands (Branden, 1980). Not only are these sites in both

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ABBREVIATIONS: CSQ, calsequestrin; SR, sarcoplasmic reticulum; cCSQ, cardiac calsequestrin; sCSQ, skeletal calsequestrin; TFP, trifluoperazine; CaM, calmodulin; SAH, serine acidic hydrophobic site; DBH, dibasic hydrophobic; ITC, isothermal titration calorimetry; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ANS, 8-anilino-1-naphthalene sulfonate; TCA, tricyclic antidepressant; CRT, calreticulin.

CSQs predicted to be binding sites from the topology diagrams, these sites also form hydrophobic grooves that are bound by exposed hydrophilic side chains, with considerable structural similarity to binding sites in other open α/β-sheet structures.

One of the phenothiazine derivatives, trifluoperazine (TFP), which is a potent inhibitor of calmodulin (CaM), has been shown to bind to a particular sequence on CaM [TFP-binding site or serine acidic hydrophobic site (SAH)], thereby inhibiting interactions between the CaM site and a dibasic hydrophobic (DBH) site on the target enzyme (Levin and Weiss, 1977; Cachia et al., 1986; He et al., 1993; Wang et al., 1998). It is now known that TFP blocks the SAH-DBH interaction by binding to a hydrophobic cleft on CaM, but not with the SAH peptide, as originally thought (Cook et al., 1994). Because the primary sequences of both cCSQ and sCSQ contain sequences similar to the SAH and DBH sites, it was suggested that there is a potential interaction between the SAH and DBH sites with unknown biological significance (Mitchell et al., 1988; He et al., 1993). TFP binding might interfere with an interaction between these two sites and thereby alter the function of CSQ (Fliegel et al., 1987). In fact, later work indicated that TFP interacts with sCSQ by an unknown mechanism, and such ligand binding evidently changes aggregation and Ca^{2+} binding properties of sCSQ (He et al., 1993).

In agreement with the prediction, the crystal structure reveals an unambiguous interaction between the putative SAH and DBH sites. This SAH-DBH interaction plays a significant role in the dimerization of CSQ molecules by forming much of the helix-helix contact in their dimer interface, rather than intramolecularly as originally proposed (He et al., 1993; Wang et al., 1998).

The unique high-capacity Ca^{2+}-binding by CSQ was also previously reported for its close association with the formation of aggregates (Saito et al., 1984; Maurer et al., 1985; Tanaka et al., 1986; Franzini-Armstrong et al., 1987; Wang et al., 1998; Park et al., 2004). Likewise, electron microscopy and cross-linking studies demonstrate that most CSQs in the SR microsomes are involved in CSQ/CSQ complexes (Saito et al., 1984; Maurer et al., 1985; Franzini-Armstrong et al., 1987; Maguire et al., 1997; MacLennan and Reithmeier, 1998) and that unique high-capacity Ca^{2+} binding of CSQ is closely associated with the formation of aggregates.

In the crystal structure of CSQ, two different types of dimers, front-to-front and back-to-back, form a continuous, linear polymer. Not only does the polymer formed by these two contacts have the linear morphology inferred for the physiologically relevant aggregation, but also the two contacts that stabilize this polymer have structural details that lend themselves to control by Ca^{2+} binding. The two dimer interfaces involve burying large surface areas and very intricate interactions, both of which are unlike typical crystal contacts. Furthermore, our studies on sequence conservation across both cCSQ and sCSQ molecules indicate that the residues involved in the back-to-back and front-to-front interfaces are the most highly conserved residues in the entire structure (Park et al., 2003). This situation is reminiscent of the higher conservation of active-site residues of other proteins and thus strongly supports our hypothesis that these dimer interfaces are the functional contacts involved in the coupled CSQ polymerization and low-affinity Ca^{2+} binding. Through chemical cross-linking, mass spectroscopy and multiangle laser light scattering, we found that at a fixed K^+ concentration of 300 mM, increasing Ca^{2+} concentration results in transitions from a single subunit to dimer and to tetramer. If Ca^{2+} is fixed, increasing K^+ concentration results in a shift toward monomers, and decreasing K^+ concentration generates polymers. These data are the first demonstrating the interplay of monovalent and divalent cations on the polymeric behavior of purified CSQ, strongly suggesting that CSQ polymerization is under dynamic regulation by mono- and divalent cations (Park et al., 2003). We have also reported a coupling mechanism between high-capacity Ca^{2+} binding and the polymerization of CSQ (Park et al., 2004).

As our knowledge of the molecular behavior of CSQs increases, we are attempting to understand the TFP binding in more detail and to identify additional compounds that may interfere with the calcium binding property of CSQ. Because CSQ is one of the major components of both cardiac and skeletal muscle and because of its key role in Ca^{2+} regulation, drug interactions with CSQ would be expected to produce adverse physiological consequences with a high frequency. Such consequences have not yet been well defined. Although undesirable drug effects or adverse drug reactions are often tolerated because of a high benefit/risk ratio, adverse drug reactions are still a major concern, and understanding the cause of these effects is important. In this regard, cardiotoxicity is a serious side effect of many drugs whose clinical usefulness is often limited by both dose- and time-dependent body accumulation and subsequent toxicity. For example, TFP is currently used as an anti-psychotic drug, but it has notorious side effects of tachycardia and muscle palpitation that often occur and persist even after its therapy is discontinued. The pathophysiologic and biochemical reasons for these side effects are not well understood, but the known interactions between TFP and CSQ suggest a likely source for adverse drug reactions.

In this article, we report investigations of the interactions between cCSQ and several pharmaceutical compounds with reported cardiotoxicity, including TFP, and the consequences of the interactions on the calcium binding capacity of cCSQ. The existence of the thioredoxin-like fold in CSQ molecules, a basic motif that often provides the platform for small molecule binding, is a reasonable target for binding by molecules such as TFP, thus causing various muscle-related side-effects. Even if the interaction between these drugs and the physiologically important protein CSQ is around the micromolar range, the accumulative effect of such interactions may produce a non-negligible problem during the course of long-term treatments. If this hypothesis is correct, then the discovery of alternative molecules with reduced affinity for CSQ provides a strategy for developing drug molecules with reduced skeletal and cardiac-related side-effects.

Materials and Methods

Plasmid Construction, Expression and Purification. Expression plasmid, pET24b-cCSQ was constructed using a polymerase chain reaction strategy. A specific region of canine cardiac calsequestrin was amplified by polymerase chain reaction using the cDNA clone as a template. A sense primer CAL5 (5′CTGTCAACATATGGAAGAGGGGCTCAACTTCCCCA3′) that contains an NdeI site and a start codon, and an antisense primer CAL3 (5′CTGATGTCGACTATTACTCATCATCATCATCACTGTCGTC3′) that contains a SalI

site followed by two stop codons were used for wild-type calsequestrin construct.

Escherichia coli strain BL21-DE3 containing plasmids with the canine cCSQ gene was grown with shaking (250 rpm) at 37°C in 1 liter of Luria-Bertani medium supplemented with kanamycin (30 μg/ml), until the optical density at 600 nm reached 0.6–0.8, at which time 0.4 mM (final concentration) isopropyl β-D-thiogalactoside was added. The culture was then shaken for an additional 3 h at 37°C and then harvested by centrifugation for 20 min at 6000*g*. The cell pellet was resuspended in 50 ml of 50 mM Tris-HCl buffer at pH 7.4. The overexpressed proteins were released from the cells by sonication (550 Sonic Dismembrator; Fisher Scientific, Pittsburgh, PA). The lysate was cleared by centrifugation at 27,000*g* for 30 min. Crude CSQ protein was separated from *E. coli* proteins by a phenyl Sepharose (Sigma) column (buffer A, 500 mM NaCl and 20 mM Tris-HCl at pH 7.5; buffer B, 20 mM $CaCl_2$, 500 mM NaCl, in 20 mM Tris-HCI at pH 7.5). The crude cCSQ was desalted and concentrated by ultrafiltration in a stirred Amicon cell with a 10,000 molecular weight cutoff membrane.

Further purification of cCSQ was carried out by anion exchange chromatography on a Mono-Q HR10/10 (Amersham Biosciences) column connected to a fast-performance liquid chromatography system. After purification, CSQ protein was desalted by ultrafiltration with a YM10 membrane with distilled water, concentrated in YM10 Centricon tubes (Millipore Corporation, Bedford, MA), and stored at 4°C for crystallization or −80°C for long-term storage. Purity of the CSQ was characterized by UV-visible absorption spectra and electrospray ionization mass spectrometry.

Isothermal Titration Calorimetry (ITC). The interactions between canine cardiac calsequestrin and different compounds were carried out on a VP-ITC instrument (MicroCal, Northampton, MA) at 25°C following standard procedures. The protein was dialyzed for 3 days at 4°C in 300 mM KCl, 0.1 mM EGTA, and 10 mM MOPS, pH 7.5. The protein was added to the calorimetric reaction cell at a concentration of 0.05 mM with 400 rpm stirring, and a syringe was used to titrate a small molecular ligand dissolved in the same buffer at a concentration of 0.5 mM into a cell containing a cCSQ solution. Compounds that did not dissolve well in dialysis buffer were first dissolved in 100% DMSO (v/v) and went through serial dilution to 2%. The protein solution was prepared to make the final concentration of DMSO in the protein the same as in the ligand solution. cCSQ protein and drug samples were degassed before use. Each titration experiment was performed with 29 injections of 10 μl at 300-s equilibration intervals. Heats of dilution for an individual ligand were determined by titrating ligand into the same buffer without protein.

Data were fit to an *n*-equivalent binding sites model at first by nonlinear least-squares regression with the Origin software package (OriginLab Corp, Northampton, MA). Because the number of binding sites (*n*) was converged to values between 0.8 and 1.2 by the initial regressions, it was fixed to 1.0 (one binding site model) in the final regressions. The fit of data yields the binding affinity, enthalpy change, entropy change, and binding stoichiometry for the titration. Compounds with positive interactions with calsequestrin were subjected to repeat experiments, and affinities for the interactions were determined by averaging results across experiments. Other compounds that did not show significant heats of reaction were not subjected to repeat experiments.

The compounds that were screened for interaction with calsequestrin were trifluoperazine, promethazine, chlorpromazine, daunorubicin, doxorubicin, 1-anilino napthalene-8-sulfonate (ANS), $NADP^+$, FAD, ATP, caffeine, guanosine, hydralazine, tetracycline, riboflavin, quinine, amitriptyline, nortriptyline, and imipramine. All compounds were purchased from Sigma (st. Louis, MO) except daunorubicin, which was purchased from Fluka Chemical Corp. (Ronkonkoma, NY).

Equilibrium Dialysis. Canine cCSQ at a protein concentration of 1–3 mg/ml was dialyzed first against distilled water followed by buffer containing a target drug (1 mM). The buffer condition for measuring Ca^{2+} binding was 10 mM Tris, pH 7.5, 300 mM KCl, and 2 mM NaN_3, which was chosen based on our CD, fluorescence spectroscopy, and light-scattering experiments (Park et al., 2003). At this condition, acquisition of secondary and tertiary structure reaches a plateau, and the molecular mass estimate from the light scattering data are 44 ± 1 kDa, corresponding closely to the molecular mass of a single CSQ molecule. Dialysis was performed for 1 week in the cold room with 10 reservoir changes. Upon completion of the buffer exchange, the dialysis bag was removed, and the protein solution was transferred to the half-cell of a modified horizontal-diffusion chamber. cCSQ solution (1.5 ml) was equilibrated against the same volume of various concentrations of $CaCl_2$ solution (0.1–10 mM) across the 12-kDa molecular cutoff dialysis membrane. Equilibrium was achieved by gentle tilt shaking for 36 h at room temperature.

Atomic Absorption Spectrophotometry. Both the protein and calcium compartments were analyzed for calcium concentration using an Atomic Absorption Spectrophotometer (Shimadzu AA-6200) at the absorption wavelength of 422.7 nm. The protein concentration of each dialysis cell was measured again by Bradford protein assay after equilibrium dialysis. Fractional occupancy (Y = [bound Ca]/[total CSQ]) was calculated as the difference in Ca^{2+} concentration between two compartments. Even though the ligand in this experiment was an electrolyte, the Donnan effect was not considered in treating the dialysis data, because the concentration of another electrolyte (KCl, 300 mM) was already high enough in both compartments.

ANS Fluorescence. The binding constant of ANS with CSQ was determined by a modified Scatchard analysis as described previously (Semisotnov et al., 1991). In brief, ANS fluorescence intensity (*F*) was collected for samples with constant CSQ protein (1 mg/ml) and increasing ANS concentration, using an excitation wavelength of 370 nm and emission value of 480 nm. A plot of 1/*F* versus 1/[ANS] gave an estimate of the *F* value as saturating ANS. At intermediate *F* values, the fraction of saturation and the amount of free protein were estimated. A Scatchard plot of (bound ANS)/(free ANS) versus (bound ANS) could then be constructed, and the minus value of the slope gave an estimate of the binding constant.

Results

Isothermal Titration Calorimetry. We performed a thermodynamic characterization of various pharmaceutical drugs that have short- and/or long-term cardiotoxicity using ITC. The major muscle-related (both skeletal and cardiac muscle) side effects of the tested drugs range from involuntary movements of muscles (dyskinesias) to arrhythmias and sudden death from heart failure. Measurement of the heat that is generated or absorbed upon ligand-binding by ITC allows accurate determination of the binding constant (K_a), enthalpy and entropy changes, thus providing a complete thermodynamic profile of the molecular interaction.

Thermodynamics values for the binding reaction are summarized in Table 1 and Fig. 1. In every case, heat was

TABLE 1
cCSQ binding parameters of drugs determined by isothermal titration calorimetry.

Drugs	K_d	ΔH	ΔS
	μM	*kcal/mol*	*cal/mol/degree*
Trifluoperazine	14	−14.6	−26.7
Promethazine	15	−12.5	−19.7
Chlorpromazine	33	−11.7	−18.6
Daunorubicin	26	−4.3	6.5
Doxorubicin	40	−3.7	7.7
Amitriptyline	15	−9.8	−10.9
Nortriptyline	11	−2.8	13.2
Imipramine	105	−1.8	−41.6

released when cCSQ associated with these compounds, indicating that the binding interactions had significant enthalpic contributions. In addition, detailed data analysis revealed slightly unfavorable entropic contributions for five of the eight molecules, possibly indicating that the CSQ structure was slightly stabilized upon binding to these five molecules.

First, we tested TFP and other phenothiazine derivatives that are being used as antipsychotic drugs: promethazine and chlorpromazine (Thorazine). TFP showed the highest affinity (14 μM), followed by promethazine (15 μM) and chlorpromazine (43 μM). Our ITC data, therefore, confirmed the previous suggestion of a CSQ-TFP interaction (He et al., 1993). These three compounds have their branched side chains on the same side of the phenothiazine ring, which was proposed to approximate the conformation of dopamine, but dopamine did not bind to cCSQ in our experiment. Neither the phenothiazine ring nor the phenazine ring alone bound cCSQ.

The phenothiazine derivatives that bound to cCSQ have high hydrophobicity, considering their reported and calculated log S (aqueous solubility) and log P (partition coefficient) values, and most of them approach the hydrophobic extreme of pharmaceutical drugs (Jorgensen, 2004). Therefore, they may well be able to penetrate into the sarcoplasmic reticulum and bind cCSQ or sCSQ.

Second, we tested tricyclic antidepressants (TCAs), such as nortriptyline (secondary amine tricyclics), amitriptyline, and imipramine (tertiary amine tricyclics). The TCAs that we tested generally showed affinity for cCSQ similar to that of the phenothiazine derivatives. Nortriptyline, amitriptyline, and imipramine had dissociation constants of 11, 15, and 77 μM, respectively. The TCAs are known to be cardiotoxic, and overdoses are frequently fatal. Electrocardiographic changes evoked by the tricyclics include QRS complex distortion, ventricular ectopic beats, nodal rhythm, bundle branch block, and ventricular tachycardia (Waterfall et al., 1979). The side effects of TCAs have been attributed to their nonspecific interaction with various unidentified proteins (Andrews and Nemeroff, 1994).

Third, we tested anthracycline derivative, daunorubicin, which is a widely used and highly effective anticancer drug. This popular anthracycline drug showed a dissociation constant of 26 μM, which is comparable with TCAs and phenothiazine derivatives. Doxorubicin also showed a considerable affinity, with a dissociation constant of 40 μM. The tetracycline ring alone showed no significant binding to cCSQ. The anthracycline derivatives are known to interfere with calcium handling mechanisms in the heart (Pessah et al., 1990; Mushlin et al., 1993; Olson et al., 2000; Burke et al., 2003), producing severe cardiotoxicity, often irreversibly damaging the heart, which somewhat limits their therapeutic potential (Olson et al., 1988). Although details of the molecular basis of these side effects are not clearly understood, especially the long-term effects after rapid and serious accumulation of the administered drug in the cardiac myocyte, evidence has been reported of possible interactions between these anthracycline molecules and the unknown components of the SR Ca^{2+} regulation complex (Zorzato et al., 1985; Pessah et al., 1990; Mushlin et al., 1993; Bowling et al., 1994).

On the other hand, other drugs that are known to have major or minor muscle-related side effects, such as cyclophosphamide, DL-isoproterenol, ephedrine, quinine, flunarizine, hydralazine, mitoxantrone, troglitazone, lonidamine, and etoposide, showed no significant binding to cCSQ. Various biochemical compounds that have a similar flat, multiring structure and similar molecular mass, such as $NADP^+$, FAD, ATP, GTP, tryptophan, and several smaller molecules, such as caffeine, adenine, guanosine, riboflavin, and quinine, also failed to show any significant binding, judging from the ITC signal. Some of typical cases of nonbinding examples are shown in Fig. 1B.

ANS Binding. ANS also binds to cCSQ. To further characterize the binding site in cCSQ, we carried out florescence spectroscopic studies with ANS (Fig. 2). Binding by this probe is relatively easy to detect because of large fluorescence changes, and this probe binds promiscuously to nucleotide, flavin, and other binding sites. ANS binding to hydrophobic patches on protein surfaces is accompanied by increases in fluorescence (typically less than 5-fold), presumably because much of the molecule remains exposed to solvent. In contrast, burying ANS in functional pockets or in the interiors of molten globules leads to 50- to 200-fold increases in fluorescence (Semisotnov et al., 1991). By this criterion, ANS seems

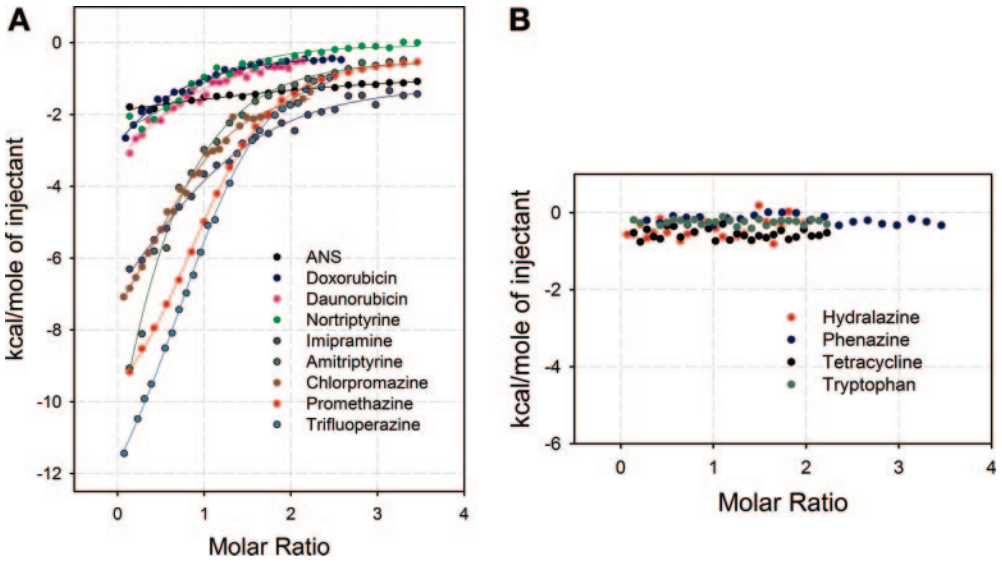

Fig. 1. The trend of heat released by serial injection of drugs into cCSQ solution. A, trifluoperazine shows the highest molar heat of binding among the compounds, and imipramine shows the lowest. The dissociation constants were calculated by curve fitting with a model equation of 'n-equivalent binding site'. B, typical heat-releasing patterns by serial injection of drugs that do not have binding affinity to cCSQ.

largely buried upon binding to CSQ because an increase in fluorescence of >50-fold is observed (Fig. 2). The binding constant of ~56 μM (Fig. 2, inset) is 2-fold tighter than the values of 100 to 300 μM observed for molten globules but within the range of 1–60 μM observed for ANS binding to various proteins having different functional pockets (Bailey et al., 2001).

Calcium Dependence of Drug Binding. To characterize the interdependency between drug binding and Ca^{2+} binding more thoroughly, cCSQ protein was pre-equilibrated with 0, 3, or 5 mM $CaCl_2$ before conducting the drug-binding calorimetric experiments. The ITC results (Fig. 3) show that the binding affinity of chlorpromazine in the presence of 3 mM $CaCl_2$ was reduced to approximately one quarter (from K_d of 33 μM to 140 μM) and was abolished almost completely in the presence of 5 mM $CaCl_2$. All of the high-affinity drugs mentioned above show a similar pattern (data not shown).

Reduction in Calcium Binding Capacity of cCSQ upon Drug Binding. Of special interest is whether the interaction of the ligand blocks high-capacity calcium binding by sterically interfering with the formation of CSQ polymer, as we suggested (Wang et al., 1998), involves substantial conformational change, or both. We performed equilibrium dialysis and atomic absorption spectroscopy to check the interference of calcium binding in the presence of drugs that show a high affinity for cCSQ.

Although the calcium binding curve of CSQ has several changes of curvature caused by the formation of various types of oligomer as the concentration of calcium increases (Park et al., 2004), the data were fitted to a simple binding model to check the reduction in calcium binding capacity caused by the interaction with the given ligand. The amount of bound calcium to CSQ was significantly reduced (Fig. 4) as the overall dissociation constant between calcium and cCSQ of 0.9 ± 0.2 mM was changed to 2.1 ± 0.3, 2.5 ± 0.4, and 2.8 ± 0.5 by promethazine, amitriptyline, and trifluoperazine, respectively, in their 1 mM concentrations. The corresponding value by tetracycline that showed no significant binding was within the error range, 1.1 ± 0.2.

Overall, the degree of inhibition for calcium binding tested by equilibrium dialysis was roughly matched with the affinity of the drug measured by ITC experiment. Trifluoperazine is the strongest inhibitor (about 60% inhibition at 1 mM concentration) of calcium binding among the compounds, and promethazine, amitriptyline, nortriptyline, and imipramine showed about 30 to 40% inhibition (at 1 mM, Fig. 5). Our experimental trials with the micromolar range of drugs were not successful, because the differences in the amount of bound calcium came close to the error range of our analysis

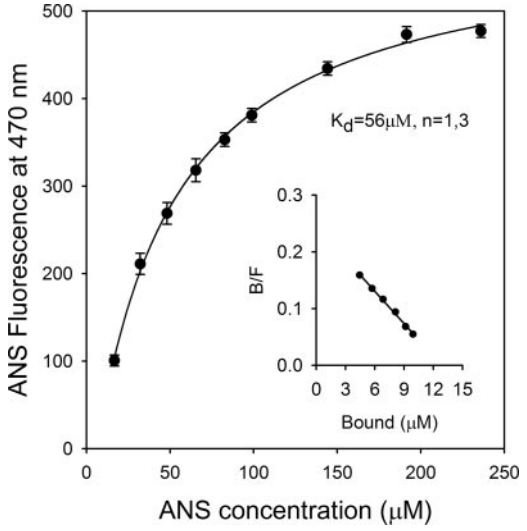

Fig. 2. ANS fluorescence. Increasing amounts of ANS were added to CSQ, and the fluorescence was recorded. From these data, a Scatchard plot was constructed (inset) as described under *Materials and Methods*; the binding constant was estimated from the minus value of the slope.

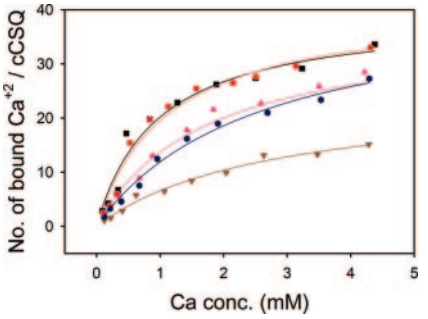

Fig. 4. Changes in calcium binding property of calsequestrin by drugs. Black square, calsequestrin only; red circle, calsequestrin with 1 mM tetracycline as a negative control; red triangle, with 1 mM promethazine, blue hexagon, with 1 mM amitriptyline; and inverted triangle, with 1 mM trifluoperazine.

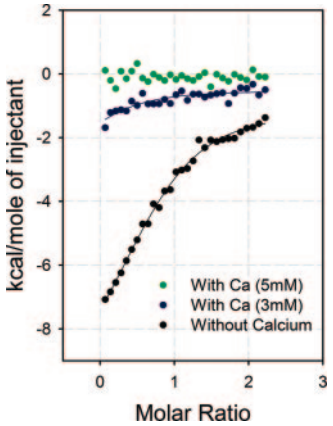

Fig. 3. Measurement of calcium dependence of drug binding through ITC experiment: pre-equilibrium of cCSQ with 3 or 5 mM $CaCl_2$ solution reduces the binding affinity of chlorpromazine

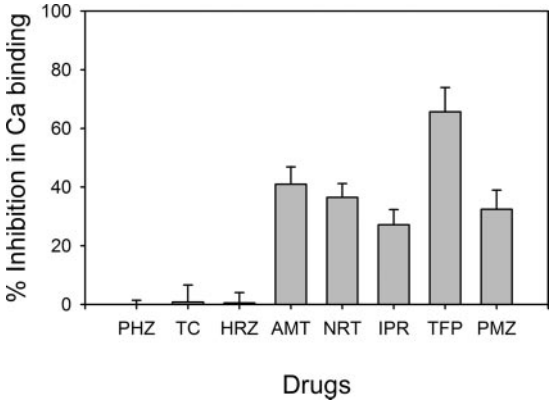

Fig. 5. Inhibition of Ca^{2+} binding by drugs (1 mM). PHZ, phenazine; TC, tetracycline; HRZ, hydralazine; AMT, amitriptyline; NRT, nortriptyline; IPR, imipramine; TFP, trifluoperazine; PMZ, promethazine.

method. We reported previously that TFP-sCSQ interaction prevented Ca^{2+}-induced aggregation of CSQ and decreased the Ca^{2+} binding by CSQ (He et al., 1993). Our result here is consistent with those earlier findings. At that time, we suggested the observed interference was caused by intramolecular SAH-DBH interactions, which we later refined by suggesting that the SAH-DBH interactions are between two different CSQ molecules rather than within one molecule and that TFP binding interferes with dimerization (Wang et al., 1998).

Discussion

The tight regulation of Ca^{2+} release to and clearance from the cytosol is essential for normal cardiac function. Catecholamine-induced polymorphic ventricular tachycardia

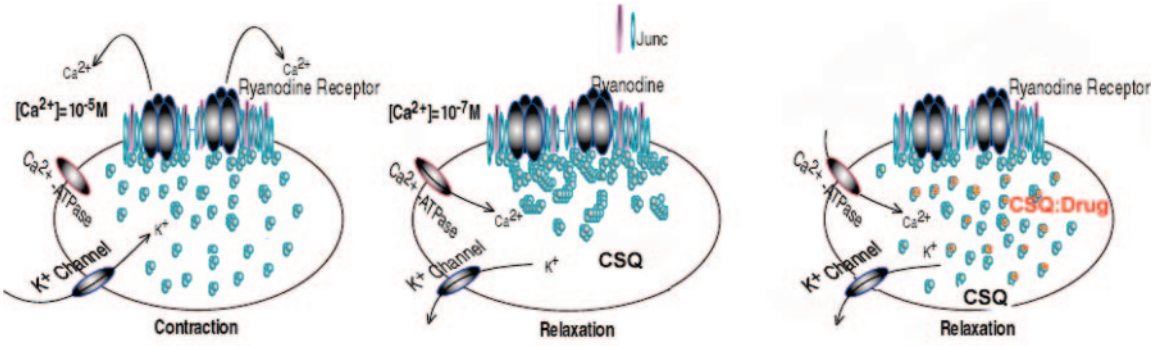

Fig. 6. Schematic diagram showing a possible mechanism of calcium uptake and release in the SR (left and center). Dynamic oligomeric status of calsequestrin at different Ca^{2+} concentrations is modulated by the opposite flux of K^+ and Ca^{2+} ions. Nonspecific ligand binding to the thioredoxin domain hinders dynamic CSQ polymerization by interfering with dimer interface formation, diminishing the Ca^{2+} binding capacities of CSQ (right).

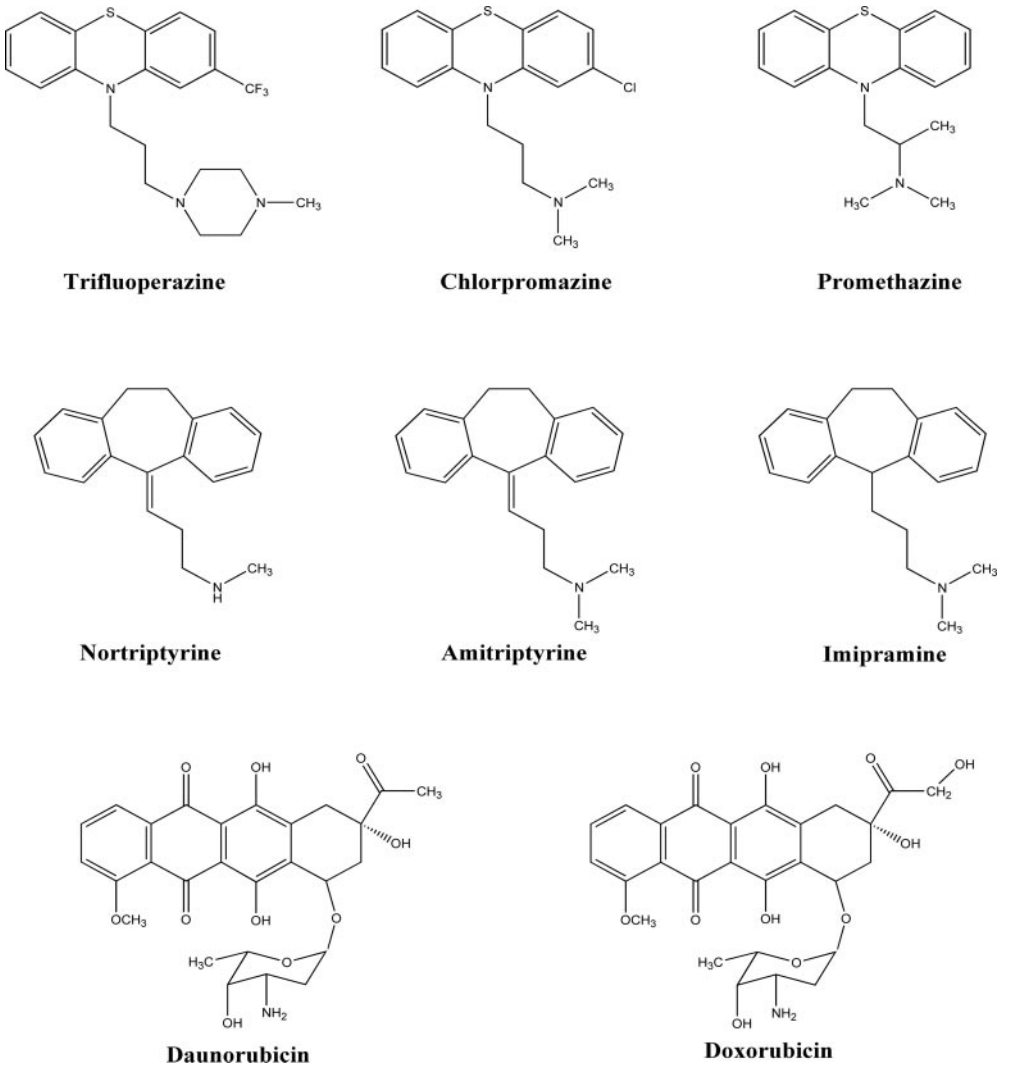

Fig. 7. Chemical structures of drugs that have an affinity to canine cCSQ.

(CPVT) is a rare arrhythmogenic disorder characterized by syncopal events and sudden cardiac death at a young age during physical or emotional stress, in the absence of structural heart disease (Viskin and Belhassen, 1998; Postma et al., 2002). Missense mutation (D307H) and nonsense mutations were found as the cause of the autosomal recessive form (Lahat et al., 2001; Eldar et al., 2003) and several additional CSQ-related CPVT families were also discovered, suggesting that mutations in cCSQ are more common than previously thought and produce a severe form of CPVT (Postma et al., 2002; Laitinen et al., 2003; Lahat et al., 2004).

In this study, we demonstrated the existence of a putative ligand-binding site in CSQ and severe interference in the Ca^{2+} binding capacity of CSQ caused by the occupancy of that site by certain kinds of small molecules, which is in accord with other known characteristics of CSQ [e.g., the individual domains of CSQ have a thioredoxin-like fold (Wang et al., 1998) and CSQ can be easily purified with an affinity column containing phenyl moiety (Mitchell et al., 1988)].

The local structure at the putative binding site in each thioredoxin-like domain of both cCSQ and sCSQ seems to be a rather deep, hydrophobic cleft that would seem to be appropriate for certain types of ligands. Many essential endoplasmic reticulum-resident proteins, such as protein disulfide isomerase and calreticulin (CRT), also contain the thioredoxin fold, judging from their amino acid sequences, and these proteins bind Ca^{2+} with low affinity and high capacity just as CSQ does. The ligand binding activities of protein disulfide isomerase and CRT have been reported previously (Nigam et al., 1994; Michalak et al., 1998; Ferrari and Soling, 1999; Corbett et al., 2000). For example, CRT binds ATP but does not exhibit detectable ATPase activity (Nigam et al., 1994; Corbett et al., 2000). The presence of three thioredoxin folds suggests that CSQ might have a previously unsuspected ligand-binding capacity and that ligand binding might play an important regulatory or adverse role in calcium regulation by CSQ.

Small hydrophobic molecules, such as phenothiazines and tricyclic antidepressants, can certainly diffuse into the sarcoplasmic reticulum (predicted log P ranges from 3.08 to 5.88 in their nonionized form) (Walter and Gutknecht, 1986) and may well accumulate there. CSQ is one of many proteins abundant (~100 mg/ml) in both skeletal and cardiac muscles, and our results show that it contains a binding site that modulates Ca^{2+} binding levels. The interactions between cCSQ and phenothiazines, TCAs, anthracyclines, and many other pharmaceutical compounds will lead to the reduction of the Ca^{2+} binding function of CSQ and consequently interfere with the normal function of the SR. It is therefore reasonable to suggest that some of the muscle-related symptoms of these drugs, such as tachycardia, may be consequences of the interaction between these drugs and CSQ.

We have proposed previously that polymerization of CSQ is directly linked to its high-capacity, low-affinity Ca^{2+} binding (Park et al., 2004). Ca^{2+} largely fills the electronegative pockets formed in the interfaces between CSQ molecules, cross-bridging the subunit and eventually forming a polymer. The propensity of Ca^{2+}-bound CSQ to form linear structures that can be formed and destroyed dynamically after the flux of K^+ and Ca^{2+} makes Ca^{2+} dissociation and diffusion a rapid event, allowing the two-dimensional diffusion of Ca^{2+} ions (Fig. 6, left and center). Small amounts of compounds that have significant CSQ binding affinity can disrupt the dynamic polymerization of CSQ by binding to the site near the SAH-DBH interaction in the dimer interface, thus hindering the high-capacity Ca^{2+} binding of CSQ and reducing the Ca^{2+}-buffering capacity of the SR (Fig. 6, right).

A significant uncertainty still exists as to whether our observation of interfering (or regulating) Ca^{2+} by binding small, hydrophobic ligands is relevant to CSQ function in vivo, because in our test, recombinant cCSQ has been used, which could disregard the importance of the post-translational modification, including a phosphorylation (Szegedi et al., 1999). In addition, the concentration of the drugs is higher than the usual tissue concentration (around the micromolar range) under the usual treatment with such drugs. However, the very high local concentrations of CSQ within the lumen of the SR means that even low concentrations of a given drug molecule (K_d in the micromolar range) could be sufficient to lead to binding and thereby to important physiological consequences. In addition, a slight alteration in the normal physiological function of the cardiac SR might be enough to cause serious problems in certain persons, and long-term exposure to such drugs could easily generate a cumulative effect (long-term toxicity). The average value of the corresponding dissociation constants listed in Table 1 (32.4 μM) gives the fractional value of cCSQ molecules occupied by the drug in tissue reaching 3~24% of the total cCSQ for the practical tissue concentration of such drugs, 1~10 μM.

The side effects of cardiac or skeletal muscle related symptoms could have several different origins such as nerve and various cellular components. In our experiments, not all the drugs having muscle-related side effects showed an interaction with cCSQ. However, given our evidence that CSQ contains a binding site for certain small molecules (Fig. 7) that alter the Ca^{2+} binding capacity, it is tempting to speculate that the devastating side effects of some pharmaceuticals, including the drugs we are reporting here, are the result, at least in part, of drug-dependent disruption of the calcium-regulating property of CSQ function in the SR. Reduced cCSQ level was associated with the accelerated Ca^{2+} discharge, and mutation of cCSQ has been suggested as a plausible explanation for ventricular arrhythmia (Terentyev et al., 2003). Considering the CSQ concentration in muscle tissue, reduced Ca^{2+} binding capacity caused by drug binding, and the tendency for long-term administration of these types of drugs, the physiological consequences of long-term treatment with such drugs that impair CSQ function are expected to be similar to those of genetic defects of this protein, such as catecholaminergic polymorphic ventricular tachycardia.

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