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Effects of Drugs with Muscle-Related Side Effects and Affinity for Calsequestrin on the Calcium Regulatory Function of Sarcoplasmic Reticulum Microsomes

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ABSTRACT

The tight regulation of Ca^{2+} release to and clearance from the cytosol is essential for normal excitation-contraction coupling in both skeletal and cardiac muscles. Calsequestrin (CSQ) is one of the major components in the sarcoplasmic reticulum (SR) of both skeletal and cardiac muscle. Previously, we showed that several pharmaceutical drugs, such as phenothiazine derivatives, tricyclic antidepressants, anthracycline derivatives, and other hydrophobic compounds bind CSQ with K_d values in the micromolar range and significantly reduce the Ca^{2+} binding capacity of cardiac CSQ (*Mol Pharmacol* **67:**97–104, 2005). Because of its key role in Ca^{2+} regulation, this interference with CSQ function could well produce adverse physiological consequences and potentially be linked to the known muscle-related side effects of these drugs. To further understand the molecular mechanism of undesirable drug effects or adverse drug reactions among those compounds, we examined their effect on the SR microsome. The results clearly showed that these compounds affect Ca^{2+} release and reduce the total Ca^{2+} content of the purified SR microsomes, matching well with our previous results with purified recombinant CSQ. Liquid chromatography-mass spectrometry/mass spectrometry showed that the antipsychotic drug trifluoperazine penetrates well into the SR microsome as expected from the reported and calculated log *S* (aqueous solubility) and log *P* (partition coefficient) values among the phenothiazine derivatives. We therefore propose that a certain portion of the muscle-related (both cardiac and skeletal) complications of these drugs is caused by the altered Ca^{2+} regulation of the SR mediated by their adverse interaction with CSQ.

The sarcoplasmic reticulum (SR) plays an essential role in muscle excitation-contraction coupling by regulating the cytosolic free Ca^{2+} concentration. The main SR proteins responsible for this Ca^{2+} regulation are the Ca^{2+} transport ATPase, the Ca^{2+} release channel ryanodine receptor, and the Ca^{2+} storage protein calsequestrin (CSQ). Among these, CSQ represents a ubiquitous, critically important Ca^{2+} storage protein found not only in the SR but also in the endoplasmic reticulum. CSQ binds 60 to 80 Ca^{2+} ions per molecule with a binding constant of approximately 1 mM under physiological conditions (MacLennan and Wong, 1971; Park et al., 2004). CSQ acts as a Ca^{2+} buffer inside the SR, lowering free Ca^{2+} concentrations in SR and thereby facilitating further uptake by the Ca^{2+}-ATPases. It also actively participates in muscle contraction by localizing Ca^{2+} at the release site and regulating the size of the functional Ca^{2+} store in SR through an interaction with the ryanodine receptor (RyR) (MacLennan et al., 2002; Terentyev et al., 2003; Beard et al., 2004).

We determined the structures of rabbit skeletal CSQ (Wang et al., 1998) and canine cardiac CSQ (Park et al., 2003). Both cardiac CSQ (cCSQ) and skeletal CSQ (sCSQ) contain three similar thioredoxin-like domains, a basic motif that often provides the platform for small-molecule binding (Branden, 1980; Katti et al., 1990; Wang et al., 1998; Park et al., 2004). Individual thioredoxin-like domain, which is composed of ~100 residues, has a five-stranded β-sheet sandwiched between four α-helices. These potential binding 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 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

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ABBREVIATIONS: SR, sarcoplasmic reticulum; TFP, trifluoperazine; CSQ, calsequestrin; DMSO, dimethyl sulfoxide; RyR, ryanodine receptor; MOPS, 4-morpholinepropanesulfonic acid; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry; c, cardiac; s, skeletal.

structural similarity to binding sites in other open-sheet structures.

One of the phenothiazine-derived antipsychotic drugs, trifluoperazine (TFP), was reported to change the conformation and Ca^{2+} binding properties of sCSQ (He et al., 1993) and to impair the normal excitation-contraction coupling of muscle (Charlier et al., 2005), reflecting its known adverse effect on normal Ca^{2+} regulation of both cardiac and skeletal muscles. In addition to their typical muscle-related side effects such as uncontrollable muscle movements (trembling and shaking), severe muscle stiffness, and spasms, TFP and other phenothiazine-based antipsychotic drugs often induce QTc prolongation (Reilly et al., 2000), polymorphic ventricular arrhythmia (Raehl et al., 1985; Witchel et al., 2003), and sudden death (Jusic and Lader, 1994; Buckley and Sanders, 2000; Reilly et al., 2000). For these reasons, intentional use of this type of drug even for suicidal purposes has become common (Choi et al., 2005).

Therefore, to further confirm the existence of a small-molecule binding site in CSQ and to investigate the consequence of binding, we carried out studies with TFP and other pharmaceuticals, such as phenothiazine derivatives, tricyclic antidepressants, and anthracycline derivatives that have reported muscle-related side effects (such as tachycardia, bradycardia, palpitation, changing P-R, QRS, QTc prolongation, heart failure, etc.) for their binding affinity to CSQ using isothermal calorimetry (Park et al., 2005). The results showed that most of the drugs which belong to phenothiazine, anthracycline, and tricyclic derivatives bind cCSQ with K_d values in the micromolar range. In most cases, binding is enthalpically driven and slightly entropically unfavorable, possibly indicating that the structure is slightly stabilized upon ligand binding. Fluorescence quenching studies also demonstrated that TFP, daunorubicin, and daunorubicinol bind to CSQ with apparent binding affinities in the micromolar range (Charlier et al., 2005). The similar binding patterns were observed for the rabbit sCSQ (E. Kim and C. Kang, unpublished data).

Upon binding, those compounds with a low K_d significantly reduced the Ca^{2+} binding capacity of cCSQ. Furthermore, fluorescence spectroscopic data for 8-anilino-1-naphthalene sulfonate binding to cCSQ closely resembles 8-anilino-1-naphthalene sulfonate binding to flavine or nucleotide-binding sites (Park et al., 2005). On the other hand, common small molecules, which have flat multiring structures, such as $NADP^+$, FAD, ATP, GTP, caffeine, adenine, guanine, tetracycline, riboflavine, and quinine, did not show any significant binding. In addition, TFP and promethazine bind cCSQ, but similarly structured thioridazine does not, indicating a potential regiospecificity in that interaction (Park et al., 2005).

When this information is combined with the high membrane permeability of these hydrophobic multiring drugs, our results lead us to the hypothesis that there could be undesirable and damaging interactions between CSQ and these pharmaceutical compounds, generating various muscle-related side effects. The very high local concentration (100 mg/ml) of CSQ within the lumen of the SR guarantees that even low blood concentrations of these drugs (approximately micromolar range for K_d values) can be sufficient to lead to binding and thereby to serious physiological consequences. A slight alteration in the normal physiological function of the cardiac SR may be enough to cause problems in many individuals. In addition, long-term treatment with such drugs could easily generate a cumulative effect, producing a non-negligible problem (long-term toxicity). Cardiotoxicity especially has been known to be a serious side effect of many pharmaceutical drugs whose clinical usefulness is often limited by both dose- and time-dependent body accumulation and subsequent toxicity.

Just like the phenothiazine-derived antipsychotic drugs, the anthracycline-derived anti-cancer drugs, such as widely used and highly effective doxorubicin and daunorubicin, produce severe cardiotoxicity, often irreversibly damaging the heart, which somewhat limits their therapeutic potential (Olson et al., 1988; Zucchi and Danesi, 2003). The pathophysiological and biochemical reasons for these side effects are not well understood. If our 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 adverse drug reaction.

Previously, we reported that binding by certain kinds of drugs significantly reduces the total amount of Ca^{2+} bound to CSQ (Park et al., 2005). However, uncertainty still exists as to whether the observation that a certain class of small, hydrophobic ligands interferes with Ca^{2+} binding by purified recombinant cCSQ is relevant to CSQ function in vivo. The experiments ignored the importance of the membrane barrier and post-translational modification of cCSQ, such as phosphorylation and glycosylation (Szegedi et al., 1999). Recently, severe inhibition of the caffeine-induced Ca^{2+} release rate from SR microsome by TFP and several other drugs, such as anthracycline derivatives, was reported (Charlier et al., 2005). Therefore, to characterize this potential interdependency between drug binding and Ca^{2+} regulation more thoroughly, we performed the experiments with the set of drugs that have a significant affinity for CSQ using purified SR microsomes.

Materials and Methods

Preparation of SR Vesicles. SR microsomes were isolated from the white skeletal muscle of rabbits by the method described previously (Saito et al., 1984; He et al., 1993; Wang et al., 1998). After removing fat and connective tissues, both back and leg muscles were ground in a meat grinder and approximately 50 g of ground meat in 250 ml of 0.3 M sucrose and 5 mM imidazole-HCl, pH 7.4, were homogenized three times for 30 s each at the maximal speed using a Waring blender (Waring Laboratory, Torrington, CT). The homogenate was centrifuged for 10 min at 7700*g*, followed by filtration of the supernatant through six to eight layers of cheesecloth. Microsomal pellets were obtained by centrifuging the supernatant for 30 min at 110,000*g*. Pellets were resuspended in 0.3 M sucrose and 5 mM imidazole-HCl, pH 7.4, and were stored at −70°C. Protein concentration was determined by the Lowry method.

SR Calcium Loading. SR microsomes were incubated for 1 h in a buffer solution (20 mM MOPS, pH 7.5, 50 mM KH_2PO_4, 5 mM KCl, 2 mM $MgCl_2$, and 2 mM ATP) with various target drugs and without drug as a control. Most of the drugs were dissolved in deionized water. Two water-insoluble compounds, L-tryptophan and tetracycline were dissolved in DMSO. After incubation, 7 nmol of $CaCl_2$ was added to the buffer containing SR microsomes to fully load Ca^{2+} into the microsome by following the method described previously (Olson et al., 2000). Quite often, more than 10 times of this loading process triggered Ca^{2+}-induced Ca^{2+} release from SR microsomes; thus, the loading was repeated up to 10 times (Olson et al., 2000). Data were

obtained through the difference absorbance spectrum (A_{710}–A_{790}) of free Ca^{2+} in solution using the metallochromic indicator antipyrylazo III and a diode array spectrophotometer (HP 8452A; Hewlett Packard, Palo Alto, CA).

Mass Spectroscopy. The amount of TFP inside of the incubated microsome was checked by modifying a method established previously (Dachtler et al., 2000). Each aliquot was acidified with 10 mM HCl for 5 min and then alkanized with 30% NH_4OH and vortexed for 5 min, followed by the additional of 1.5% isoamyl alcohol in heptane. The sample was then centrifuged at 1000*g*, and the heptane layer was collected repeatedly. The combined heptane extracts were evaporated to dryness with a SpeedVac (Thermo Electron, Waltham, MA) and reconstituted with 30 μl of liquid chromatography mobile phase (35% 20 mM ammonium acetate, 0.035% acetic acid, and 65% of acetonitrile). LC-MS/MS was performed on an Agilent-1100 series LC system (Agilent Technologies, Palo Alto, CA) with Microbore C_{18} column (Micro-Tech Scientific Inc., Vista, CA) coupled to an API 4000 triple quadruple mass spectrometer with electrospray ionization source (Applied Biosystems, Foster City, CA). The mass spectrometer was operating in selective reaction monitoring mode, observing parent ion of 408.1 Da and product ion of 113.2 Da (Fig. 2). The sample and calibration standards were run in triplicate.

Atomic Absorption Spectrophotometry. The total amount of calcium in the SR microsomes was analyzed using an Atomic Absorption Spectrophotometer (Shimadzu, Kyoto, Japan) at the absorption wavelength of 422.7 nm. After ultracentrifugation, the SR microsome pellet was dissolved in HCl overnight and then vaporized in a flame. Calcium concentration was determined after calibrating the instrument with a series of standard solutions of $CaCl_2$ (Fluka Chemical Corp., Ronkonkoma, NY). Protein content for individual pellets was assayed by the Lowry method to normalize calcium content.

Calcium Release Effect of TFP. The Ca^{2+} was loaded to SR microsomes following the 10-step process, as described above. To investigate the effect of TFP, the final Ca^{2+} loading step was immediately followed by adding TFP to a final concentration of 0.2 mM, and the Ca^{2+} release was measured by monitoring the absorbance changes of 710 and 790 nm through the diode array spectrophotometer (HP 8452A). To check whether the observed TFP-induced Ca^{2+} release can be blocked by an RyR channel blocker, ruthenium red was added to the solution containing Ca^{2+}-loaded SR microsomes to a final concentration of 10 or 20 μM at 20 s after the application of TFP.

Results

To determine whether a given ligand that has a significant binding affinity to CSQ and reduces the Ca^{2+}-binding

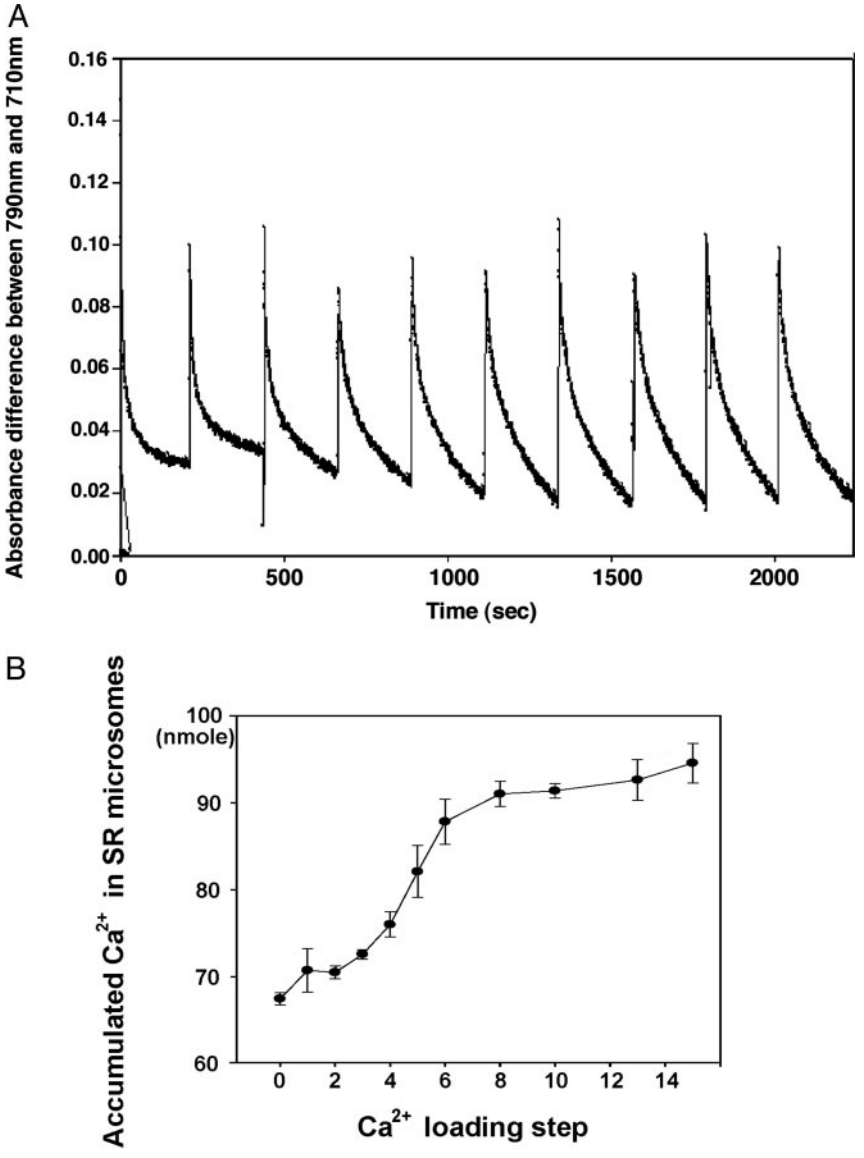

Fig. 1. A, representative tracing of the process of loading SR microsomes with Ca^{2+}. The metallochromic indicator, antipyrylazo III, was used to monitor calcium uptake by measuring differences in absorbance (A_{710}–A_{790}), using an HP8452A UV/Vis diode-array spectrophotometer. B, The amount of calcium in SR microsomes at each $CaCl_2$ loading step. With continuous mixing, 7 nmol of $CaCl_2$ was added to the 1-ml buffer solution containing ~5 mg SR microsomes. After 5 min of each loading, the SR solution was ultracentrifuged, and the amount of Ca^{2+} was measured through Atomic Absorption Spectrophotometer (Shimadzu) at the absorption wavelength of 422.7 nm.

capacity of CSQ affects the total Ca^{2+} amount of SR microsomes, we performed ATP-dependent Ca^{2+} loading into purified rabbit skeletal SR microsomes. Each 7 nmol Ca^{2+} addition to the buffer that contains SR microsomes shows similar absorbance changes up to ~10 times loading (Fig. 1A). As shown in Fig. 1B of the Atomic Absorption Spectrophotometer data, the total amount of calcium in the SR microsomes was gradually increased reaching a plateau. This indicates that Ca-ATPase functioned properly to uptake Ca^{2+} into the lumen of SR microsome, and abnormal Ca^{2+} leakage out of SR microsome did not occur during the Ca^{2+} loading experiment. Sets of parallel SR calcium-loading experiments were performed with and without drugs that have the potential to cause short- and/or long-term cardiotoxicity, such as the following: 1) phenothiazine derivatives, including TFP, promethazine, and chlorpromazine; 2) tricyclic antidepressants, including nortriptyline, amitrityline, and imipramine; and 3) two anthracycline-based anticancer drugs, daunorubicin and doxorubicin. All of these compounds have micromolar range affinity to cCSQ and an inhibitory effect on its calcium binding capacity (Park et al., 2005). The potential (skeletal and cardiac) muscle-related side effects of these drugs range from involuntary movements of muscles (dyskinesias) to arrhythmias and sudden death from heart failure.

Effect of Diffused Drugs on Ca^{2+} Storage Capacity of the SR Microsome. To study the effect of the cCSQ-binding drugs on the Ca^{2+} storage capacity of SR microsomes, microsomes were preincubated for 1 h with 1 mM concentration of either cCSQ binding or control drugs.

First, to quantify the amount of drugs that diffused into the SR microsomes, LC-MS/MS was performed for one of the target drugs, TFP (Fig. 2). The data show that the treated SR microsomes holds 1.02 ± 0.02 μg of TFP per 1 mg of SR microsomes that was incubated for 1 h in a buffer containing 1 mM TFP (Table 1). Even after rinsing the microsome pellet with fresh buffer twice, the SR microsome still contains 0.75 μg of TFP per 1 mg of SR microsomal pellet (Table 1).

After the 1-h incubation with and without drugs, 10 times Ca^{2+} loading was done as described under *Materials and Methods*. After this Ca^{2+} loading, each tube was immediately ultracentrifuged, and the SR pellet was incubated in HCl until it completely dissolved. The total amount of calcium in each tube was analyzed by atomic

TABLE 1
TFP quantification in SR pellet after incubation with 1 mM TFP through LC-MS/MS

Sample	TFP amount
SR pellet after TFP incubation	1.02 ± 0.02 μg of TFP/mg of SR pellet
Supernatant after ultracentrifugation	11 ± 2 μg of TFP/ml of supernatant
SR pellet after rinsing with a fresh buffer	0.75 ± 0.05 μg of TFP/mg of SR pellet
Supernatant after reultracentrafugation	1.4 ± 0.2 μg of TFP/ml of supernatant

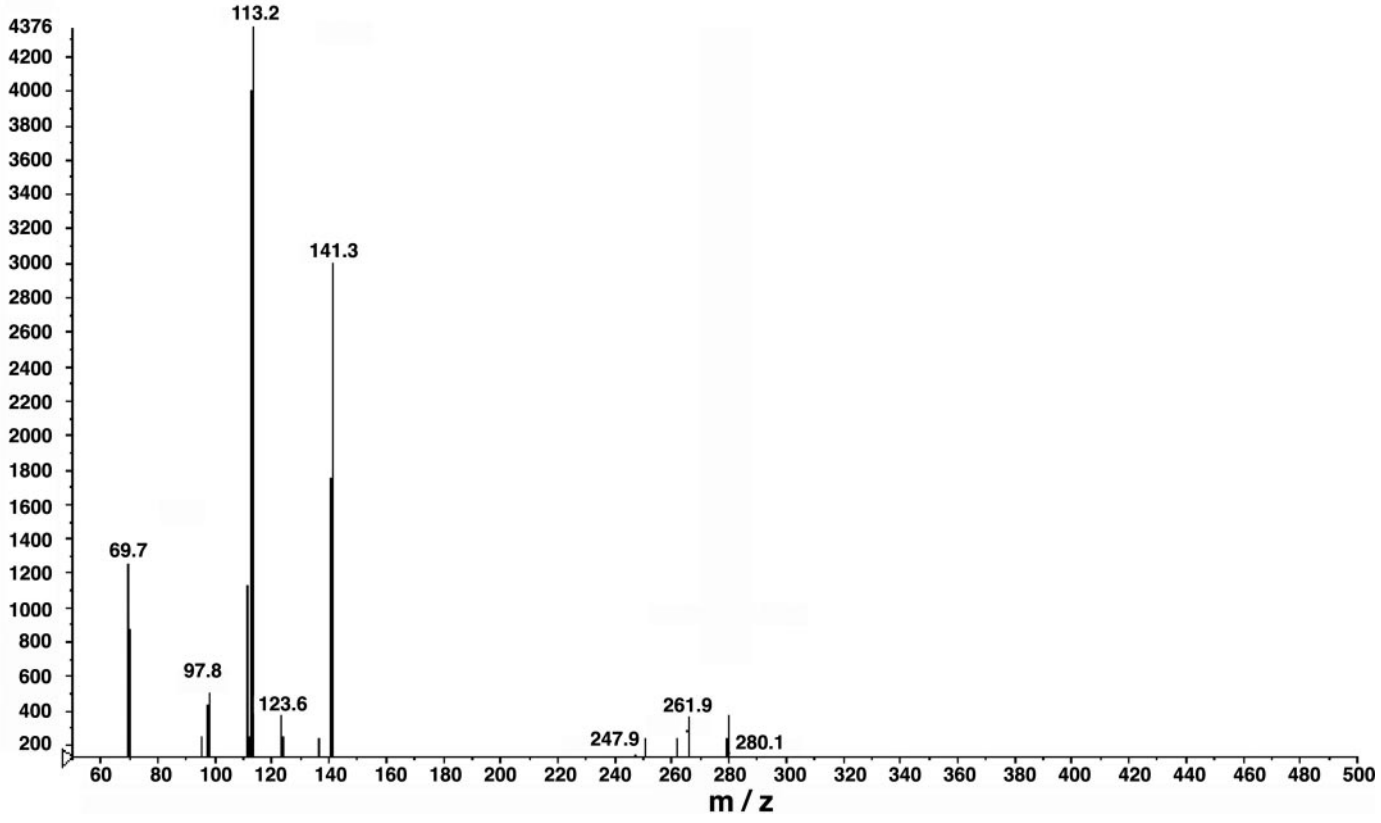

Fig. 2. Mass spectroscopic data. MS/MS product ion spectrum of TFP (100 nM) under the conditions of the analysis, showing fragments of the protonated parent ion selected by Q1 (408.1 *m/z*, entirely consumed in collision process). Calibration of TFP by LC-MS/MS was done in selective reaction monitoring mode.

absorption spectroscopy; Table 2 and Fig. 3 show the normalized values. The total Ca^{2+} content of the tube, of which SR microsomes was loaded with Ca^{2+} (10 times) in the absence of any drug, was considered to be 100% for each set of experiments. As shown in Table 2 and Fig. 3, in every case, the SR microsome that was exposed to the CSQ binding drugs showed a significantly lower level of total calcium content compared with that of Ca^{2+}-loaded SR microsomes without drug preincubation. Among the tested drugs, TFP is the strongest inhibitor (~40% inhibition at 1 mM concentration) of calcium storage capacity, which is somewhat in agreement with our previous results by isothermal titration calorimetry (Park et al., 2005). This degree of inhibition by TFP is followed, in decreasing order, by doxorubicin, promethazine, daunorubicin, amitriptyline, nortriptyline, chlorpromazine, and imipramine. Most of these CSQ-binding drugs showed an inhibition level of ~30 to 40%. In contrast, other compounds that have no binding affinity for CSQ despite their reported major or minor muscle-related side effects, such as ephedrine, theophylline, flunarizine, and dopamine, did not show any significant effect on the SR calcium contents. Ephedrine, theophylline, dopamine, and the solvent for dissolving a water-insoluble drug, DMSO, showed even slightly increased Ca^{2+} values. In addition to these, various biochemical compounds that have a similar flat multiring structure and molecular weight, such as tetracycline, L-tryptophan, and several smaller-sized molecules such as guanosine, riboflavine, and quinine did not show any significant effect on the total Ca^{2+} content of SR microsome (data not shown).

TABLE 2

Ca^{2+} content of SR after incubation with drugs and after 10 times of Ca^{2+} loading (with each step of 7 nmol of $CaCl_2$ to the 1 ml of buffer solution containing ~33 mg of SR microsomes)

Rabbit skeletal sarcoplasmic reticulum (33 mg/ml) dissolved in a buffer solution (20 mM MOPS, pH 7.5, 50 mM KH_2PO_4, 5 mM KCl, 2 mM $MgCl_2$, and 2 mM ATP) was incubated with 1 mM concentration of each drug for 1 h and titrated 10 times with 7 nmol of $CaCl_2$.

Drugs	Ca^{2+} Capacity of SR
	%
Original SR	77.94
Ca^{2+}-added SR	100
Ephedrine	100.58
Theophylline	100.88
Flunarizine	97.37
Dopamine	100.08
Tetracycline	93.94
DMSO	102.0
L-Tryptophan	92.39
Imipramine	67.07
Daunorubicin	61.75
Doxorubicin	61.02
Chlorpromazine	64.07
Nortriptyline	62.5
Amitriptyline	62.16
Trifluoperazine	59.75
Promethazine	61.42

Concentration and Time Dependence of the Drug Effect on SR Calcium Content. The cCSQ binding drug, TFP, also showed concentration and time dependencies in its effect on total calcium contents of the SR microsome (Figs. 4 and 5). For the concentration dependence study, TFP concentration in the buffer containing microsomes was varied from 10 μM to 1 mM, and as a negative control, the cCSQ nonbinding drug ephedrine was used. Figure 4 shows that the microsome incubated for 1 h in a buffer containing 10 μM

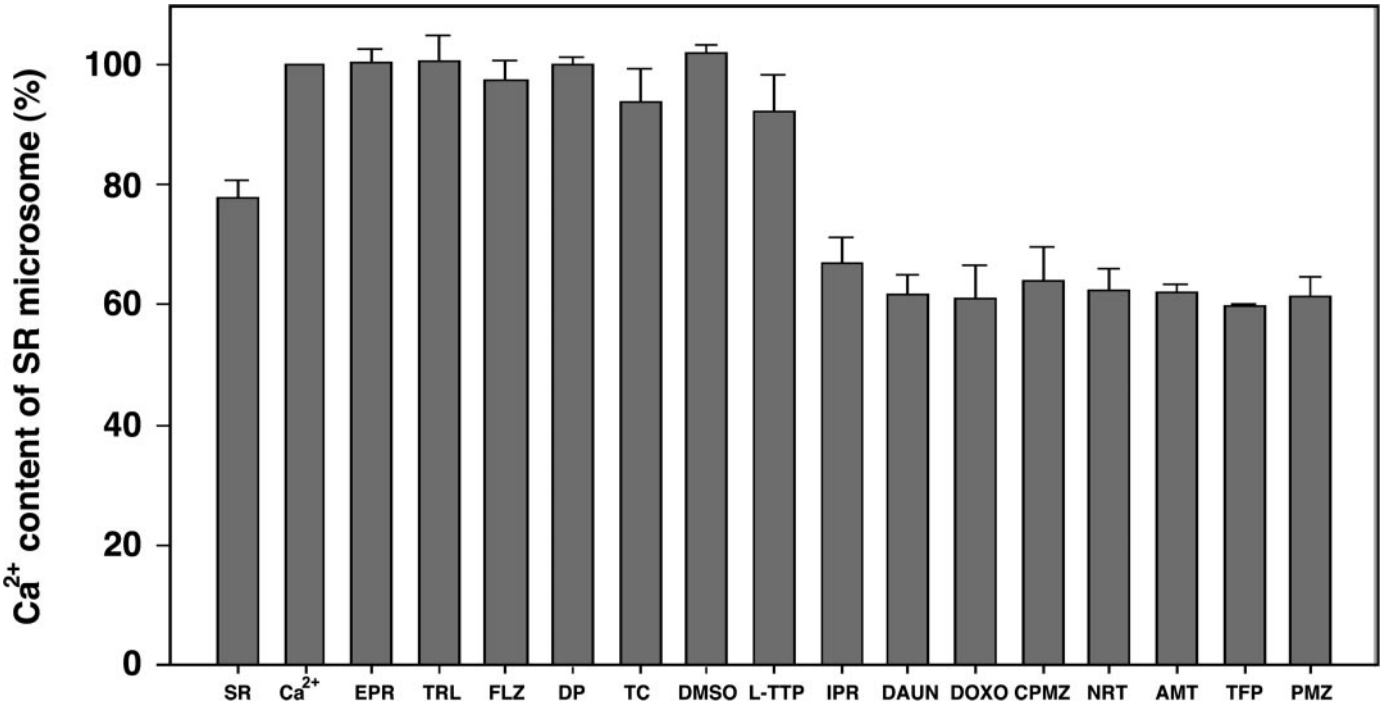

Fig. 3. Inhibition effect of certain drugs that have muscle-related side effects. SR microsomes were incubated in a buffer containing each drug and were loaded 10 times with Ca^{2+}. The Ca^{2+} content for each sample was measured through atomic absorption spectroscopy. Data are expressed as a percentage of the amount of Ca^{2+} in drug-untreated SR microsome. SR, original SR microsome without any Ca^{2+} or drug; Ca^{2+}, Ca^{2+} loaded SR microsome without any drug; EPR, ephedrine; TPL, theophylline; FLZ, flunarizine; DP, dopamine; TC, tetracycline; L-TTP: L-tryptophan; IPR, imipramine; DAUN, daunorubicin, DOXO, doxorubicin, CPMZ, chlorpromazine; NRT, nortriptyline; AMT, amitriptyline; TFP, trifluopromazine; PMZ, promethazine.

TFP does not produce any significant difference in calcium content compared with the control SR. However, as the TFP concentration was increased, the calcium content of the SR microsomes was drastically reduced. A similar pattern of TFP concentration dependence was observed for the Ca^{2+}-binding capacity of purified recombinant canine cCSQ (Park et al., 2005). As expected, ephedrine, which has no significant affinity to cCSQ, shows no effect on the total calcium content of SR microsome in all concentration ranges tested.

For the incubation-time dependence of TFP, SR micro-

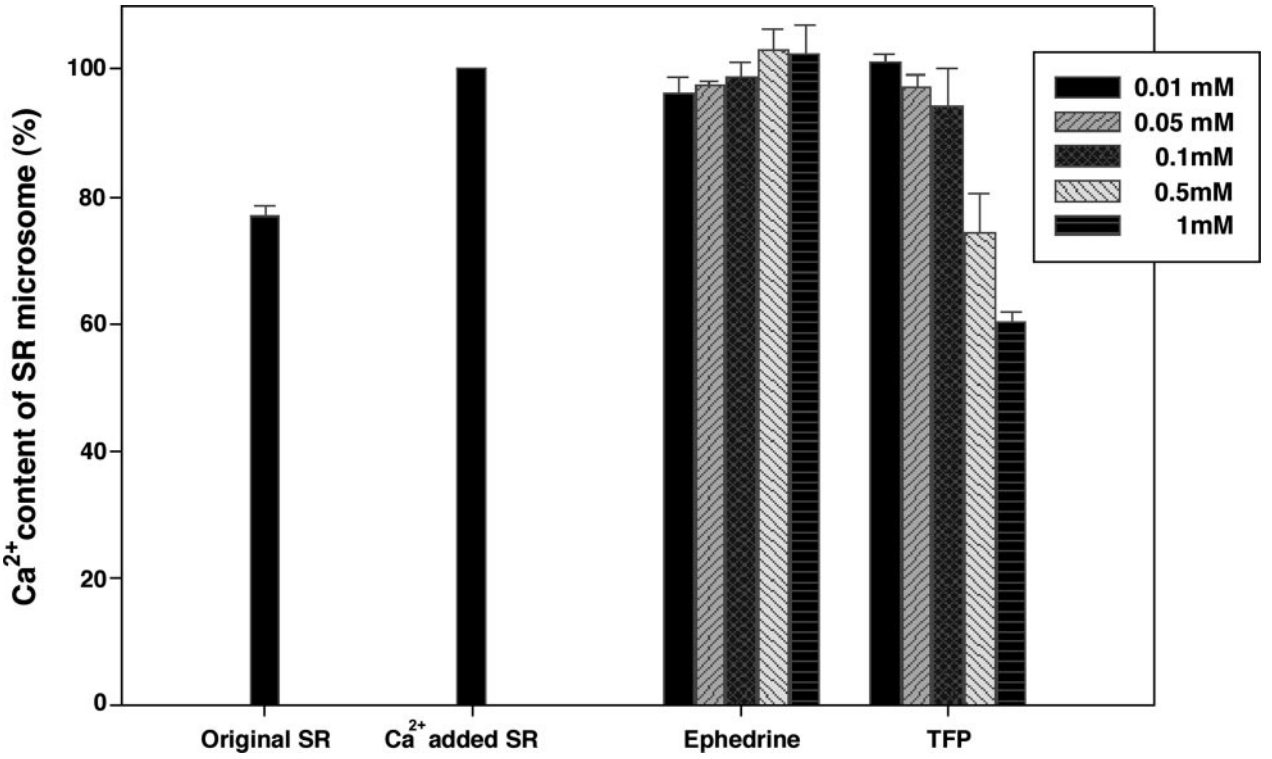

Fig. 4. Concentration dependence of TFP inhibitory effect. SR microsomes were incubated in the presence of TFP and ephedrine before the addition of Ca^{2+}. The concentrations of two drugs were varied from 0.01 to 1 mM.

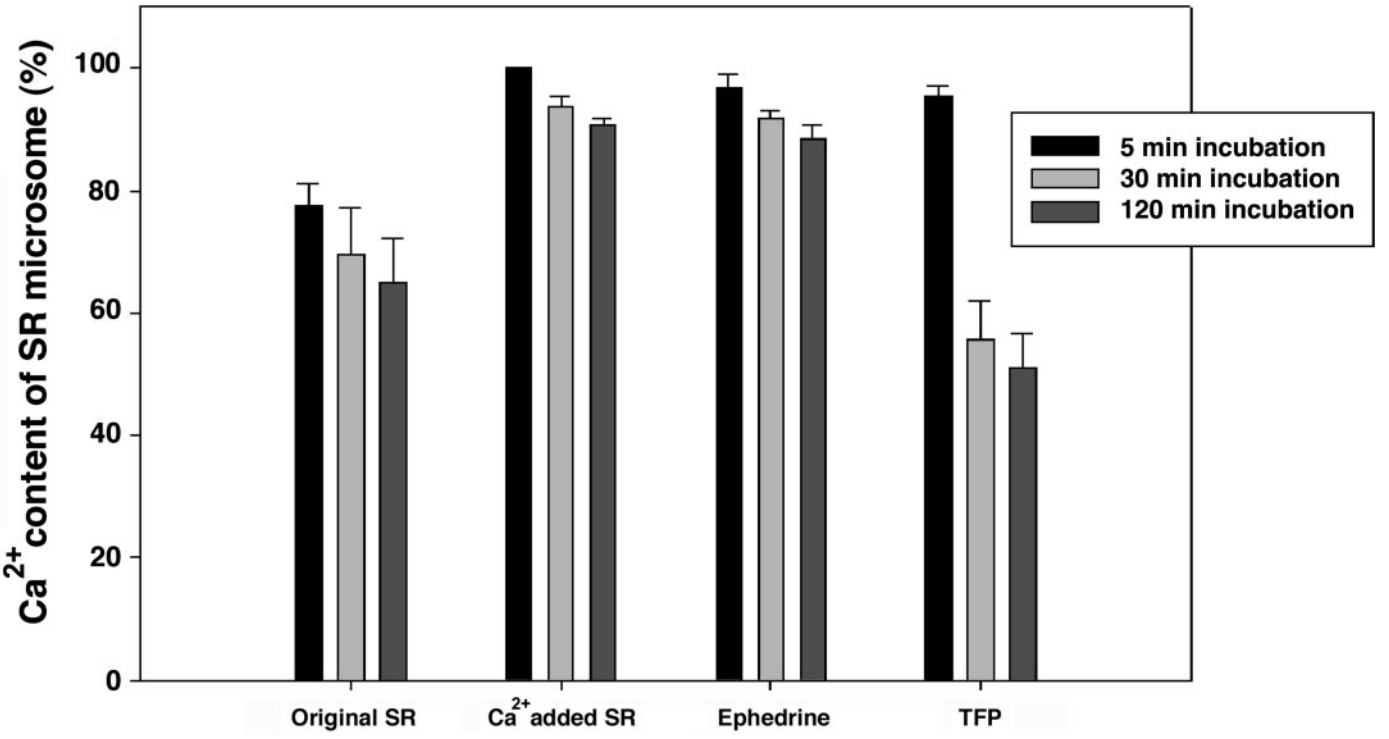

Fig. 5. Time dependence of TFP. Total calcium contents were measured for SR microsomes that were incubated in a loading buffer containing 1 mM concentration of TFP or ephedrine for 5, 30, and 120 min.

somes were incubated in buffer containing 1 mM TFP for 5, 30, and 120 min. A gradual decrease in calcium content was observed in all samples, possibly caused by long incubation in the presence of continuous mixing the solution with a magnetic stirrer at room temperature. As incubation time increased, the Ca^{2+} content of the SR preincubated with TFP decreased significantly (Fig. 5). After 120-min preincubation with TFP, the SR had almost 50% of the Ca^{2+} content of the control SR. In the case of ephedrine, the reduction in Ca^{2+} content with increased preincubation time was similar to that of the control SR.

Effect on Calcium Release. As shown in Fig. 6, data using antipyrylazo III and the diode array spectrophotometer indicate that the CSQ binding drug TFP is able to release Ca^{2+} from microsomes (▽), even though the control microsomes continuously take Ca^{2+} from the buffer in the presence of ATP (descending black circle line at the bottom of the figure). By adding a channel blocker for RyR, ruthenium red, we were able to inhibit this drug-induced Ca^{2+} release in a concentration-dependent manner (■ and ◇). Therefore, the TFP-induced release of calcium is, at least in part, thorough the RyR1.

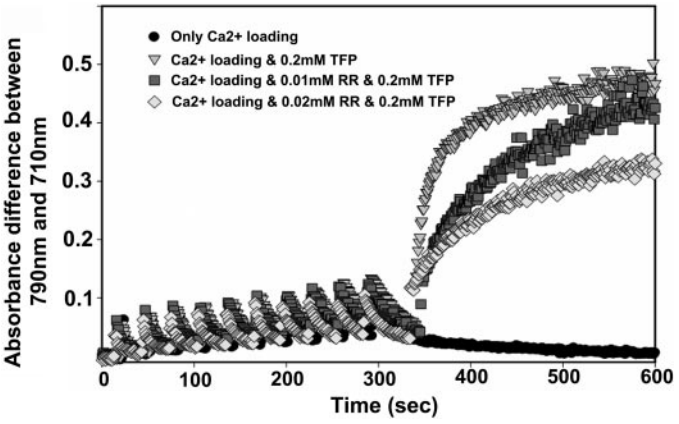

Fig. 6. Calcium release effect of TFP. For the upper trace (▽), TFP (0.2 mM final concentration) was added to SR microsomes after the 10th loading of Ca^{2+}. The bottom trace (●) shows absorbance changes after the addition of the 10th loading of Ca^{2+}. The two middle traces show absorbance changes after the 10th loading of Ca^{2+} followed by addition of ruthenium red and TFP, with final concentrations of 10 μM (■) and 20 μM (◇), respectively, and 0.2 mM TFP.

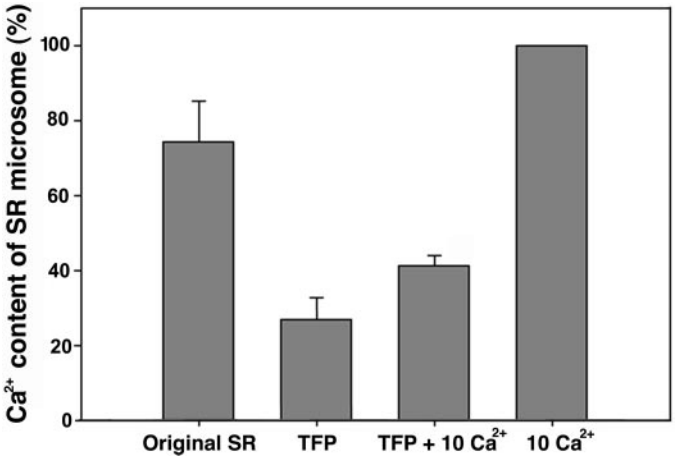

Fig. 7. The effect of absorbed TFP. SR microsomes were incubated for 1 h in a loading buffer containing 1 mM concentration of TFP. Then they were centrifuged and rinsed twice to remove the unincorporated TFP, and the pelleted SR microsomes were redissolved in fresh buffer without TFP. While continuously mixing the solution with a magnetic stirrer, Ca^{2+} was loaded 10 times by adding 7 nmol of $CaCl_2$ solution each time, which elevated the calcium concentration of the solution to 70 nM. Original SR, SR without any Ca^{2+} or drug treatment; TFP, TFP preincubated SR without Ca^{2+} loading; TFP + 10 Ca^{2+}, TFP preincubated and 10 times Ca^{2+} loaded SR; 10 Ca^{2+}, 10 times Ca^{2+} loaded SR.

To observe the effect of TFP that is already inside of the SR microsome, each SR microsome was pelleted after incubation with and without TFP for 1 h. The microsomes were further rinsed twice to completely remove the unincorporated TFP. The rinsed pellets were immediately redissolved with calcium-loading buffer, and the calcium-loading experiment was performed as described above. As shown in Fig. 7, the calcium content of the TFP-treated SR was much less than that of the untreated microsome. Even after a 10× calcium load, the SR still did not reach the original calcium level; hence, penetrated TFP molecules result in unrecoverable reduction of the total storage capacity of the SR.

Discussion

Our mass spectroscopy data show that small hydrophobic molecules, such as phenothiazine derivatives, can rapidly diffuse into SR. This behavior among phenothiazine and anthracycline derivatives is consistent with their reported and calculated log *S* (aqueous solubility) and log *P* (partition coefficient) values. Most of these compounds approach the hydrophobic extreme of pharmaceutical drugs (Walter and Gutknecht, 1986). Therefore, considering our LC-MS/MS data and previous isothermal calorimetry data (Park et al., 2005), those penetrated drugs will be trapped in SR. Given the high concentration of CSQ in SR and the micromolar range affinity between cCSQ and this class of pharmaceutical drugs (Park et al., 2005), long-term and/or high-dose administration of these drugs will certainly lead to accumulation of these compounds in the SR. Previously, we showed that the normal Ca^{2+}-binding capacity of purified CSQ is reduced in a concentration-dependent manner by binding to these classes of drugs (Park et al., 2005). Therefore, the accumulation of drugs affects both the buffering capacity and the storage level of Ca^{2+} inside of the SR, which is an important determinant of functional activity of RyR.

CSQ serves as a luminal Ca^{2+} sensor through dynamic affinity with the RyR complex. At low Ca^{2+} concentration in the SR, CSQ inhibits the activity of the RyR channel complex through physical interaction. Upon increasing the luminal concentration of Ca^{2+}, this inhibitory interaction is gradually relieved, increasing the probability of channel opening (Terentyev et al., 2003; Beard et al., 2004; Gyorke et al., 2004).

It is tempting, therefore, to speculate that binding of drugs such as TFP to CSQ weakens the physical interaction between CSQ and the RyR complex, relieving the inhibitory role of CSQ for the RyR and increasing the opening rate of RyR. In addition, a decreased buffering capacity of CSQ and the luminal SR will result in a higher concentration of the free luminal Ca^{2+} during a period of Ca^{2+} re-entry by the Ca^{2+} ATPase; thereby, a large fraction of RyRs would be in a CSQ-uninhibited mode. This, in turn, accelerates Ca^{2+} discharge.

In this way, exposure to the CSQ-binding drugs can produce a rapid leakage of Ca^{2+}, as observed here and previ-

ously (Wykovsky et al., 1988; Olson et al., 2000), and can lower the total Ca^{2+} storage level of the SR in both short- and long-term ways, depending on the level of exposure. We therefore propose that some of the muscle-related side effects among the pharmaceutical drugs, including ones used in this report, from a trembling or shaking to a more serious arrhythmia and sudden cardiac arrest, are the consequence, at least in part, of the interaction between the diffused drugs and CSQ. The reduction of the functional CSQ will produce the same side effects, such as tachycardia, that are observed in the cases of genetic impairment of cCSQ and down-regulation of cCSQ (Terentyev et al., 2003).

The exact nature of the conformational change induced by drug binding is still not clear, even though we have proposed a hypothetical model before (Park et al., 2005). Elucidation of molecular details will show how certain drugs interact with CSQ, and a clear understanding of the underlying regiochemical and stereochemical conformation responsible for drug binding is likely to provide new and global insights into avoiding various types of harmful drug interactions. The studies derived from the corollary hypothesis using SR microsome or CSQ provide not only a potentially simple method for screening drugs but also ideas of how to redesign many pharmaceutical drugs with notorious side effects.

Of course, many reported drug side effects of cardiac or skeletal muscle-related symptoms could have several origins. As noticed herein and in our previous experiments, not all of the drugs with reported muscle-related side effects showed an interaction with cCSQ (Park et al., 2005) nor an effect on the calcium-regulating function of the SR.

Noticeably, many endoplasmic reticulum resident proteins such as protein disulfide isomerase and calreticulin, also contain the thioredoxin fold to which every domain of CSQ belongs. These proteins bind Ca^{2+} with low affinity and high capacity, just as CSQ does. Unusual ligand binding activities among these proteins have been reported previously (Nigam et al., 1994; Michalak et al., 1998; Ferrari and Soling, 1999; Corbett et al., 2000). The presence of a thioredoxin fold in these essential regulatory proteins suggests that ligand binding to these proteins may well affect their role in calcium regulation, producing a wide variety of side effects. Therefore, the physiological and biomedical relevance of the adverse interaction between thioredoxin folds and certain classes of drugs is probably very high.

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