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Investigations of Calsequestrin as a Target for Anthracyclines: Comparison of Functional Effects of Daunorubicin, Daunorubicinol, and Trifluoperazine

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ABSTRACT

Anthracycline therapy is associated with a life-threatening but poorly understood cardiotoxicity. Effects of treatment are consistent with drug-induced disruption of cardiac sarcoplasmic reticulum (SR) calcium homeostasis, including inhibition of calcium release by anthracyclines. This effect, which depends on luminal SR calcium concentration, is hypothesized to involve interactions of anthracyclines with the calcium binding protein calsequestrin (CSQ). This study was designed to test the hypothesis that an interaction between CSQ and anthracyclines could be related to alterations in SR calcium release and cardiac function. The effects of the anthracycline, daunorubicin, and its metabolite daunorubicinol were compared with those of a known CSQ inhibitor, trifluoperazine (TFP). Protein fluorescence quenching studies demonstrated that TFP, daunorubicin, and daunorubicinol bind to CSQ with apparent binding affinities in the low micromolar range. The presence of calcium decreases the drug-dependent fluorescence quenching, probably because of calcium-induced CSQ conformational changes. TFP also inhibited SR calcium release. Although the TFP IC_{50} value is somewhat larger than for anthracyclines, the TFP effect is also dependent on luminal SR calcium concentration. In a muscle preparation, micromolar TFP decreased cardiac contractility in a manner that implicates the involvement of SR calcium and resembles the effects of anthracyclines. These data are consistent with a mechanism in which TFP or anthracyclines impair SR calcium release and cardiac function through a mechanism involving disruption of CSQ function. Such a mechanism may contribute to anthracycline cardiotoxicity.

Anthracyclines are effective anticancer drugs whose use is limited by a chronic cardiotoxicity for which the mechanism is poorly understood (Zucchi and Danesi, 2003). The effects of anthracycline treatment are consistent with disruption of cardiac Ca^{2+} homeostasis (Cusack et al., 1993b; Arai et al., 1998; Matsushita et al., 2000; Shadle et al., 2000; Wang et al., 2001). Anthracyclines, such as doxorubicin and daunorubicin, and their metabolites doxorubicinol and daunorubicinol, respectively (Fig. 1A), alter sarcoplasmic reticulum (SR) Ca^{2+} regulation (Pessah et al., 1990; Mushlin et al., 1993; Olson et al., 2000; Shadle et al., 2000). The metabolites are important because they accumulate in heart tissue (Cusack et al., 1993b; Stewart et al., 1993) and perturb cardiac function to a greater extent than the parent (Cusack et al., 1993a; Olson et al., 2000).

One important effect is anthracycline-induced inhibition of SR Ca^{2+} release (Olson et al., 2000). Cardiac SR vesicles preincubated with daunorubicin or daunorubicinol before Ca^{2+} loading exhibit reduced caffeine-induced Ca^{2+} release rates. The earlier daunorubicin or daunorubicinol is added

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ABBREVIATIONS: SR, sarcoplasmic reticulum; CSQ, calsequestrin; TFP, trifluoperazine; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ANOVA, analysis of variance; LSD, least significant difference; PRP30, post–rest-potentiated/30-s rest interval; RyR2, type 2 ryanodine receptor.

during the SR Ca^{2+} loading process, the greater the inhibition of release (Olson et al., 2000). This is potentially important because it involves clinically relevant concentrations (nanomolar to low micromolar) of anthracyclines and their metabolites (Cusack et al., 1993a; Stewart et al., 1993). The effect is in contrast to that of micromolar anthracyclines, which induce Ca^{2+} release from Ca^{2+}-loaded vesicles via a mechanism analogous to that of caffeine (Pessah et al., 1990; Olson et al., 2000; Shadle et al., 2000).

The mechanism for SR Ca^{2+} release inhibition by anthracyclines has not previously been investigated. However, the dependence of inhibition on Ca^{2+} loading suggests the anthracycline interacts with a protein whose function in Ca^{2+} regulation/release depends on luminal SR Ca^{2+}. A candidate target is calsequestrin (CSQ), which has recently been shown to bind anthracyclines (Park et al., 2005). Although chronic anthracycline treatment alters CSQ mRNA levels (Arai et al., 1998), CSQ-anthracycline interactions have not been linked to functional cardiotoxic effects.

CSQ is a luminal protein of the SR that functions in sequestration of Ca^{2+}, control of intracellular calcium stores, and regulation of SR Ca^{2+} release (Terentyev et al., 2003; Györke et al., 2004; Park et al., 2005). CSQ binds Ca^{2+} with low affinity (K_d = ~0.5–1 mM) and high capacity (~20–80 Ca^{2+}/CSQ) and undergoes significant Ca^{2+}-dependent conformational changes (Slupsky et al., 1987; Mitchell et al., 1988; Park et al., 2005). Its high-capacity Ca^{2+} binding is thought to involve protein aggregation (Gatti et al., 2001; Park et al., 2003). Its regulatory function probably requires the formation of a complex with the RyR2 calcium release channel and the transmembrane proteins triadin and junctin (Györke et al., 2004). Crystallographic results show three thioredoxin-like folds, which are proposed to bind hydrophobic ligands (Wang et al., 1998; Kang, 2001; Park et al., 2005).

Although the effects on cardiac function from small molecule-mediated inhibition of CSQ activity are unknown, the importance of CSQ for normal cardiac function has been demonstrated. Transgenic mice overexpressing CSQ exhibit cardiac hypertrophy and changes in cardiac function (Knollman et al., 2000; Sato et al., 2003). A missense mutation in the CSQ gene is a cause of catecholaminergic polymorphic ventricular tachycardia and is related to disruption of CSQ function (Viatchenko-Karpinski et al., 2004). Reduction in CSQ levels decreases SR Ca^{2+} stores and the magnitude and duration of Ca^{2+} transients, and alters Ca^{2+} sparks (Terentyev et al., 2003).

Because the inhibition of SR Ca^{2+} release by anthracyclines suggests the possible involvement of CSQ, this study was designed to test the hypothesis that an interaction between CSQ and anthracyclines could be related to alterations in SR Ca^{2+} release and cardiac function. The effects of daunorubicin and daunorubicinol were compared with those of a known CSQ inhibitor, trifluoperazine (TFP). TFP (Fig. 1B) is a calmodulin inhibitor that binds to CSQ and inhibits CSQ Ca^{2+} binding (Park et al., 2005), conformational changes, and aggregation (He et al., 1993). Furthermore, TFP inhibits these functions only if it is added to the protein before Ca^{2+} (He et al., 1993), an effect that resembles the Ca^{2+} load dependence of SR Ca^{2+} release inhibition by anthracyclines (Olson et al., 2000).

In this study, the binding of TFP, daunorubicin, and daunorubicinol to CSQ was investigated using protein fluorescence quenching. Isolated SR and atrial muscle preparations were used to study the ability of TFP to inhibit SR Ca^{2+} release and alter cardiac contractility, respectively. The effects of TFP are compared with those of daunorubicin and daunorubicinol from previous experiments (Olson et al., 2000; Shadle et al., 2000). The results provide insight into a mechanism in which anthracycline-dependent inhibition of SR Ca^{2+} release and disruption of cardiac function might be mediated by a direct interaction with CSQ.

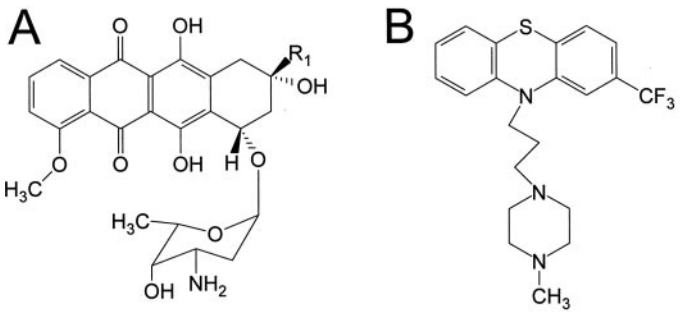

Fig. 1. Structures of daunorubicin [R_1, **C**(=O)CH_3] and its primary metabolite, daunorubicinol [R_1, **C**H(OH)CH_3] (A) and TFP (B). The bold carbon in the R1 groups of daunorubicin and daunorubicinol is numbered C13.

Materials and Methods

Materials. Daunorubicin, TFP, and all other chemicals, unless otherwise noted, were obtained from Sigma-Aldrich (St. Louis, MO). Daunorubicinol was synthesized according to published methods (Takanashi and Bachur, 1976). Canine cardiac CSQ was overexpressed in *Escherichia coli* and purified according to established procedures (Kobayashi et al., 2000). Purified samples were concentrated and exchanged into 20 mM MOPS, pH 7.2, 150 mM NaCl using an Amicon Ultrafiltration 8050 stirred cell (Millipore Corporation, Billerica, MA) and stored at 4°C until use. Experiments with animals were conducted in accordance with the Declaration of Helsinki and the Guide for Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

CSQ Fluorescence Quenching Studies. Fluorescence measurements (λ_{ex} = 275 nm, λ_{em} = 331 nm) were made using a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA). Procedures were performed at 23°C. A sample containing 7.5 μg/ml canine cardiac CSQ in 20 mM MOPS, pH 7.2, 150 mM NaCl was brought to a final concentration of 0 or 2.2 mM Ca^{2+} by the addition of water or $CaCl_2$. CSQ samples to which Ca^{2+} was not added showed no detectable presence of Ca^{2+}, as measured by atomic absorption (SolAAr M; Thermo Electron Corporation, Waltham, MA), for which the lower detection limit is ~2.5 μM Ca^{2+}. Vehicle (water), daunorubicin, daunorubicinol, or TFP was titrated into the reaction with stirring.

Relative fluorescence data, F/F_0 were fit to the Stern-Volmer equation (eq. 1) using NONLIN (Johnson and Frasier, 1985), in which Q is the concentration of the quencher, f_1 is the fraction of protein fluorescence that is quenched, and K_d is the dissociation constant for CSQ and drug. Fitted values were compared using a Student's t test.

$$\frac{F}{F_0} = \frac{f_1}{1 + Q/K_d} + (1 - f_1) \quad (1)$$

Preparation of Cardiac SR Vesicles. Canine cardiac SR vesicles were prepared and stored as reported previously (Shadle et al., 2000). Protein concentrations were determined as described by Lowry et al. (1951) using bovine serum albumin as the standard.

Calcium Release Studies. The metallochromic indicator antipyrylazo III was used to measure Ca^{2+} release by measuring the

difference in absorbance between 710 and 790 nm (A_{710}–A_{790}) using an HP 8450A UV/Vis diode array spectrophotometer (Hewlett Packard, Avondale, PA) (Palade and Vergra, 1982). Procedures were performed at 32°C. One-milliliter assays consisted of 0.34 mg of protein/ml cardiac canine microsomes in 0.30 mM antipyrylazo III, 20 mM MOPS, 50 mM KH_2PO_4, 5 mM KCl, 2 mM $MgCl_2$, and 2 mM ATP, pH 7.0. Thereafter, calcium chloride (7 nmol) was added to load the microsomes with calcium; the process was repeated until the microsomes were loaded with 10 aliquots of calcium. The loaded microsomes were then exposed to 6 mM caffeine, and rates of calcium release (nanomoles of Ca^{2+} per milligram of protein per minute) were determined spectrophotometrically. Some experiments included the addition of the SR calcium pump inhibitor cyclopiazonic acid (4.5 μM) after calcium loading was complete, but before the addition of caffeine.

Data were analyzed as follows to determine the rate of release expressed in nanomoles of Ca^{2+} per minute per milligram. Antipyrylazo III requires an internal calibration for the relationship between nanomoles of Ca^{2+} and absorbance (Scarpa et al., 1978). This was achieved by averaging the increase in the absorbance difference (A_{710}–A_{790}) for each of 10 loads for all experiments to obtain an extinction coefficient for the absorbance difference (A_{710}–A_{790}) for Ca^{2+} of 0.00340/nmol/cm. The average extinction coefficient determined from control experiment loads was the same as that from TFP-treated loads. Thus, data from all experiments were averaged for the determination of the extinction coefficient. The release rates were determined from the initial slope of each progress curve after addition of caffeine. All release rates for TFP-treated samples are reported as percentages of control release rates

Assay of SR Ca^{2+} Release in Microsomes Preincubated with TFP. Effects of preincubating SR microsomes with TFP on caffeine-induced Ca^{2+} release were studied in isolated SR preparations. The results were compared with those from microsomes preincubated with the TFP vehicle (water). The microsomes (340 μg) were incubated (32°C) in 1 to 60 μM TFP or vehicle for several minutes before starting Ca^{2+} loading (10 loads of 7 nmol of Ca^{2+}/load). Experiments using higher concentrations of TFP were not performed because of an inability to fully load the vesicles with Ca^{2+}. In other studies, TFP or vehicle was added after the seventh Ca^{2+} load. Ca^{2+} efflux rates (nanomoles of Ca^{2+} per minute per milligram) as a result of addition of 6 mM caffeine were determined. Mean release rate values from experiments where TFP was added before Ca^{2+} loading or after the seventh load were compared using a Student's unpaired t test (two-tailed). The control experiments using TFP vehicle added at zero loads or after the seventh load showed no difference between zero (N = 9) and seventh (N = 6) load (data not shown). An overall average of the control experiments was used in the analysis.

Muscle Function Studies. Studies of rabbit cardiac muscle function were conducted as described previously (Shadle et al., 2000). Contractility, or the maximal rate of rise of force (dF/dt; grams per second), was obtained for each atrial preparation at contraction frequencies of 1, 2, and 3 Hz (1 Hz = 1 beat/s) and for the first contraction after a rest interval of 30 s (PRP30). After obtaining baseline values, TFP was added to achieve cumulative concentrations of 100, 200, or 300 μM in the buffer. Atrial preparations were incubated for 60 min at each cumulative concentration. Control samples showed no significant changes in measured contractility (dF/dt) over the time course of the experiment. Data were analyzed for significant differences using ANOVA with repeated measures and Fisher's LSD method.

Computational Modeling. TFP and daunorubicin structures were built in Gaussian 98 (Frisch et al., 1998) and optimized using Hartree-Fock theory and the 6-31G basis set. The minimized drug structures were converted to a protein database file type and transferred to Spartan 2.0. In Spartan 2.0, an equilibrium geometry optimization was performed for each molecule using a Hartree-Fock 3-21G* basis set, and a surface of density potential was built. The highest resolution was chosen for rendering the final illustrations shown in Fig. 8.

Results

Experimental Strategy. Anthracycline-dependent inhibition of SR Ca^{2+} release and disruption of cardiac function suggest that anthracyclines may act via a mechanism in which the anthracyclines target CSQ. This hypothesis was tested by comparing the actions of anthracyclines with those of the CSQ inhibitor TFP. If anthracyclines are also CSQ inhibitors, then their effects should resemble those of TFP. In particular, this study was designed to investigate the CSQ binding properties of anthracyclines, their metabolites, and TFP as well as the ability of these drugs to inhibit Ca^{2+} release from the SR and to perturb cardiac function in a similar manner.

TFP, Daunorubicin, and Daunorubicinol Bind to CSQ. Fluorescence quenching studies were used to investigate the binding of daunorubicin, daunorubicinol, and TFP to CSQ. In these experiments, TFP was titrated into a buffered CSQ solution in the presence of 0, 1, or 2.2 mM Ca^{2+}. Luminal SR Ca^{2+} is buffered near 1 mM Ca^{2+} (Park et al., 2005). In studies investigating Ca^{2+}-dependent CSQ fluorescence changes, 2.2 mM Ca^{2+} falls in a concentration range in which protein fluorescence is no longer altered by addition of Ca^{2+} (Slupsky et al., 1987; Mitchell et al., 1988). Furthermore, the 2.2 mM Ca^{2+} conditions allow us to probe binding to a more aggregated form of the protein (Park et al., 2003). Our results show that TFP quenches CSQ protein fluorescence in a concentration-dependent manner (Fig. 2A). Fits of these data to the Stern-Volmer equation (eq. 1) provided estimates of the apparent dissociation binding constant, K_d, and the maximal

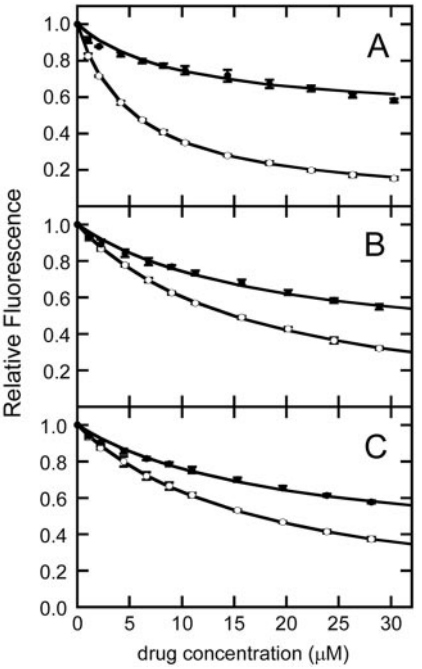

Fig. 2. Relative CSQ protein fluorescence (λ_{ex} = 275 nm, λ_{em} = 330 nm) in the absence (○) and presence (●) of 2.2 mM Ca^{2+} as a function of added TFP (A), daunorubicin (B), and daunorubicinol (C). Experiments conducted at 1 mM Ca^{2+} are similar to those at 2.2 mM Ca^{2+} (Table 1). Data are averages of three experiments; error bars show S.E.M. Lines show fits to the data with eq. 1.

quenching reached at saturation, f_1 (Table 1; Fig. 2A). Preequilibration of CSQ with 1 mM Ca^{2+} modestly decreases the TFP binding affinity to CSQ; additional 2.2 mM Ca^{2+} does not alter K_d further. Furthermore, the extent of fluorescence quenching (f_1) was dependent on the presence of Ca^{2+}. TFP quenches CSQ fluorescence to a significantly greater extent in the absence of Ca^{2+} than in the presence of 1 mM Ca^{2+} ($p < 0.001$). The difference in f_1 suggests that a greater fraction of tryptophans is protected from fluorescence quenching by TFP in the presence of Ca^{2+}. There is no substantial difference in binding or quenching between 1 and 2.2 mM Ca^{2+}.

Because both daunorubicin and daunorubicinol are present in heart tissue after anthracycline treatment (Cusack et al., 1993b; Stewart et al., 1993), the interaction of each molecule with CSQ was investigated. Both daunorubicin and daunorubicinol quench CSQ fluorescence in a concentration-dependent manner (Figs. 2B and 4C). Daunorubicin binds with similar affinity in both the absence and presence of Ca^{2+} (Table 1). The K_d values reflect approximately 2- or 3-fold weaker binding for daunorubicin to CSQ, compared with TFP. Like TFP, the extent of protein fluorescence quenching (f_1) depends on the presence of Ca^{2+} (Table 1). As with TFP, protein fluorescence quenching by daunorubicin is more pronounced in the absence of Ca^{2+} than in the presence of 1 mM Ca^{2+} ($p < 0.001$). Daunorubicinol binds to CSQ and quenches its fluorescence ± Ca^{2+} in a manner almost identical to that of daunorubicin (Table 1). Thus, the oxidation state of the C13 carbon (Fig. 1) of daunorubicin/daunorubicinol does not seem to influence binding interactions with CSQ.

Caffeine-Induced Calcium Release Is Inhibited by TFP and Is Dependent on Luminal Ca^{2+} Concentration. The ability of TFP to inhibit SR Ca^{2+} release from isolated SR vesicles was investigated to probe the effect that an interaction with CSQ might have on calcium regulation and to compare the effects to those of anthracyclines. Experiments were conducted in which SR vesicles were preincubated with TFP and then loaded 10 times with Ca^{2+} (7 nmol of Ca^{2+}/load), followed by addition of caffeine to initiate Ca^{2+} release. Low concentrations of TFP (1 μM) increase the rate of Ca^{2+} release slightly, whereas at higher concentrations (≥10 μM), TFP inhibits the rate of Ca^{2+} release significantly (Fig. 3). At 60 μM TFP, the calcium release rate is only 30% that of the control. The concentration required to decrease SR Ca^{2+} release to rates 50% that of control (IC_{50}) was 40 μM (Table 2). To verify that this effect was caused by alterations in calcium release only, control experiments were conducted in which the SR calcium pump inhibitor cyclopiazonic acid was added after calcium loading was complete, followed by the addition of caffeine to elicit release. The results of these control experiments (data not shown) were not different from those shown in Fig. 3, indicating that the inhibition is related primarily to an effect on SR calcium release.

In an effort to further characterize the inhibitory effect, we investigated whether exposure to TFP before or after Ca^{2+} loading would influence inhibition of Ca^{2+} release. The data demonstrate that inhibition of caffeine-induced Ca^{2+} release is dependent upon when TFP was added during the Ca^{2+} loading process (Fig. 4). Addition of 50 μM TFP before loading results in release rates that are only 40% of control rates. In contrast, TFP added after the seventh Ca^{2+} load results in reduced release rates that are 68% of control rates ($p < 0.05$ compared with experiments with TFP added before loading) (Table 2).

Table 2 also summarizes results of similar experiments conducted previously with daunorubicin and daunorubicinol (Olson et al., 2000). Comparison shows that IC_{50} values for daunorubicin and daunorubicinol (1.2 and 0.6 μM, respec-

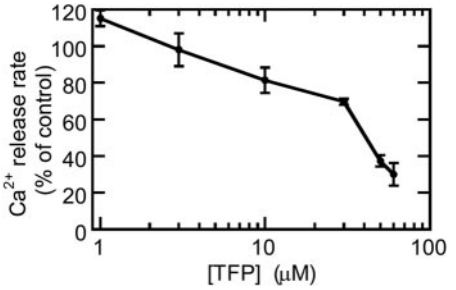

Fig. 3. Concentration dependence of TFP-dependent inhibition of caffeine-induced SR Ca^{2+} release rates. Data show release rates in response to added 6 mM caffeine as a percentage of control vehicle values. Values are mean ± S.E.M. of three experiments at each concentration, except for 0, 1, 10, and 60 μM TFP, N = 9, 4, 4, and 2, respectively.

TABLE 1

Parameters for TFP and anthracycline binding to CSQ

Protein (7.5 μg/ml) in 20 mM MOPS, 150 mM NaCl, pH 7.2, in the presence of 0, 1, or 2.2 mM Ca^{2+}, was titrated with drug, and the quenching of CSQ fluorescence was determined with excitation at 275 nm and emission at 331 nm. The fluorescence quenching data were fit to the Stern-Volmer equation (eq. 1). K_d is the dissociation constant for drug dissociation from CSQ and f_1 is the fractional protein fluorescence. Drug concentrations ranged from 0 to 35 μM. Values in parentheses are fitting errors.

Drug	[Ca^{2+}]	K_d	f_1
	mM	*μM*	
TFP	0.0	5.4 (0.2)	0.99 (0.01)
	1.0	10 (3)	0.56 (0.07)
	2.2	10 (2)	0.51 (0.03)
Daunorubicin	0.0	17 (1)	1.08 (0.02)
	1.0	18 (2)	0.73 (0.06)
	2.2	20 (3)	0.72 (0.05)
Daunorubicinol	0.0	18 (1)	1.03 (0.03)
	1.0	17 (2)	0.80 (0.05)
	2.2	20 (2)	0.71 (0.05)

TABLE 2

Effects of TFP and anthracyclines on SR Ca^{2+} release

In control experiments, canine cardiac sarcoplasmic reticulum vesicles (0.34 mg of protein/ml) in 0.30 mM antipyrylazo III, 20 mM MOPS, pH 7.0, 50 mM KH_2PO_4, 5 mM KCl, 2 mM $MgCl_2$, and 2 mM ATP were loaded with Ca^{2+} (7 nmol/load). In drug experiments, TFP or anthracycline was added to the microsomes before Ca^{2+} loading or after the sixth or seventh load. In both drug and control experiments, Ca^{2+} efflux rates (nanomoles of Ca^{2+} per minute per milligram) as a result of the addition of 6 mM caffeine were determined spectrophotometrically. A total of 10 Ca^{2+} loads were used in the TFP experiments; 11 loads were used in the anthracycline experiments. Concentrations of drug used in Ca^{2+} load dependence studies were 50 μM TFP and 1 μM daunorubicin and daunorubicinol.

Drug	Dose Dependence IC_{50}	Ca^{2+} Load Dependence	
		Load at Which Drug Was Added	Observed Release Rate
	μM		*% of control*
TFP	40	0	40
		7	68
Daunorubicin[a]	1.2	0	56
		6	68
Daunorubicinol[a]	0.6	0	43
		6	62

[a] Data from Olson et al. (2000).

tively) are lower than for TFP. However, the TFP concentration dependence, including the small increase in the caffeine-induced release rate seen at low concentrations of TFP, is qualitatively similar to that observed in experiments in which vesicles were preincubated with anthracyclines and Ca^{2+} release was induced with caffeine (Olson et al., 2000). Furthermore, the calcium load dependence of TFP inhibition shows a pattern very similar to that of daunorubicin and daunorubicinol. When TFP or anthracycline concentrations were chosen so that the release rate values are in the range of 40 to 60% of control (for drug added before any Ca^{2+} loading), then incubation with TFP or anthracycline after the sixth or seventh Ca^{2+} load causes rates that were ~62 to 68% of control (Table 2). Thus, the effects of the CSQ inhibitor TFP on Ca^{2+} release show inhibition trends similar to those of daunorubicin and daunorubicinol. These results provide evidence to support the hypothesis that CSQ could be a target for anthracyclines and that this interaction could impair SR Ca^{2+} homeostasis.

Cardiac Function Is Impaired by TFP and Is Related to the SR. The ability of TFP to alter cardiac function was investigated to allow for a comparison with the effects of anthracyclines. The effects of TFP on contractility (dF/dt) of rabbit heart atrial preparations, as a function of contraction rate, are shown in Fig. 5. Data show that TFP decreased contractility at all rates of contraction in a dose-dependent manner. However, higher rates of contraction were selectively affected. At 200 μM, TFP causes significant inhibition of contractility at both 2 and 3 Hz, compared with controls. At 100 μM, TFP causes significant inhibition of contractility only at 3 Hz, compared with controls. Contractility (dF/dt) at higher rates of contraction depends more on Ca^{2+} from SR than other Ca^{2+} sites (Janczewski and Lewartowski, 1986; Bouchard and Bose, 1989). Thus, these results suggest the TFP-dependent inhibitory effects on contractility involve changes to the regulation of SR Ca^{2+}.

TFP also decreases contractility (dF/dt) of both steady-state (1 Hz) and rest-potentiated contractions, the initial contractions after a 30-s rest interval (rested contraction, PRP30), in a dose-dependent manner (Fig. 6). However, changes in rest-potentiated contractions demonstrate a selective effect. At 100 μM TFP, neither type of contraction is different from controls. At 200 μM, only the contractility of PRP30 preparations is significantly inhibited compared with controls. TFP at 300 μM inhibits both steady-state and PRP30 contractions. Because the ability of a rest interval to augment contractility depends more on SR Ca^{2+} than on Ca^{2+} from other sites (Bers, 1985; Sutko et al., 1986; Bose et al., 1988; Bouchard and Bose, 1989; Bouchard et al., 1989), these results also suggest the TFP-dependent inhibition observed in these experiments involves changes to the regulation of SR Ca^{2+}.

Analogous experiments, performed in our laboratory and designed to evaluate the effects of daunorubicin on cardiac contractility, yielded results similar to those observed with TFP (Shadle et al., 2000). For daunorubicin-treated rabbit atrial preparations, contractility of post-rest–potentiated contractions (20-, 30-, and 60-s rest intervals) is significantly inhibited compared with control, whereas there is no effect on steady-state (1 beat/s) contractility. For example, contractility of PRP30 contractions is 75 and 55% of control for 88 and 175 μM daunorubicin, respectively (Shadle et al., 2000). In addition, contractility of contractions at 2 and 3 beats/s is significantly inhibited compared with steady state (1 beat/s). At 88 μM daunorubicin, contractility was 75 and 65% of control at 2 and 3 beats/s, respectively; at 175 μM daunoru-

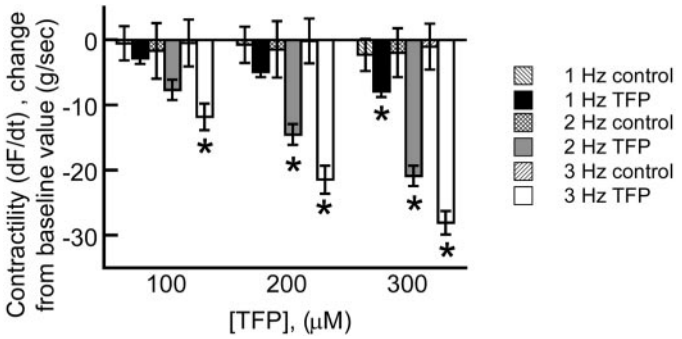

Fig. 5. Differences in contractility (dF/dt) as a function of buffered TFP concentration in isolated atrial preparations from adult rabbits. Values were obtained at contraction rates of 1, 2, and 3 Hz. Data are averages of $N = 7$ separate TFP experiments and $N = 3$ separate control experiments; error bars show S.E.M. (*, $p < 0.05$ compared with difference in control; one-way ANOVA with repeated measures and Fisher's LSD method).

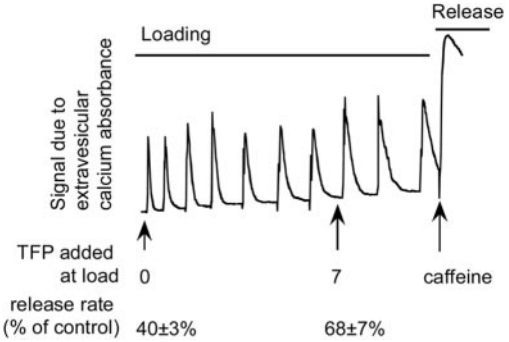

Fig. 4. TFP-dependent inhibition of caffeine-induced Ca^{2+} release as a function of Ca^{2+} load. The representative tracing of the SR Ca^{2+} release experiment shows the extravesicular Ca^{2+} absorbance, indicative of 10 loads of Ca^{2+} and the caffeine-induced release. TFP (50 μM) was added to SR vesicles before addition of Ca^{2+} (zero load) or after the seventh Ca^{2+} load. Release rates were determined from the initial slope of the progress curve after addition of caffeine. Release rate values (mean ± S.E.M.) are expressed as a percentage of control (TFP vehicle) experiments. TFP release rate values are an average of $N = 3$ experiments.

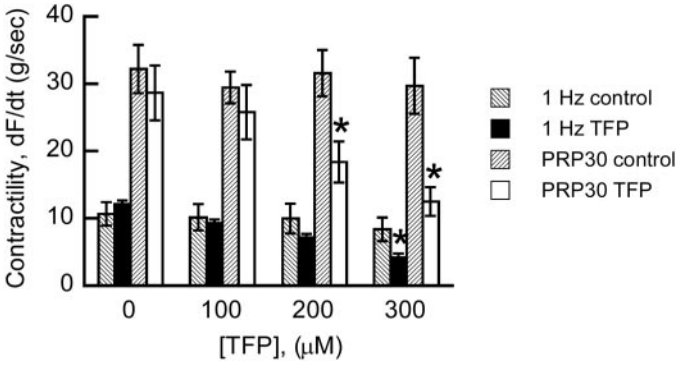

Fig. 6. Contractility (dF/dt) of post–rest-potentiated (PRP) contractions compared with steady-state (ss) contractions (1 Hz) as a function of TFP concentration for isolated atrial preparations from adult rabbits. Rest interval was 30 s for PRP contractions. Data are averages of $N = 7$ separate TFP experiments and $N = 3$ separate control experiments; error bars show S.E.M. (*, $p < 0.05$ compared with control values; one-way ANOVA with repeated measures and Fisher's LSD method).

bicin, contractility was 45 and 35% of control at 2 and 3 beats/s, respectively. At higher concentrations (350 μM daunorubicin), steady-state contractility is also inhibited. Both of these effects of daunorubicin on contractility are concentration dependent (Shadle et al., 2000). Thus, similar to TFP, the inhibitory effects of daunorubicin on contractility are dependent on the rate of contraction and rest interval, implicating an SR Ca^{2+}-dependent mechanism, one that may involve CSQ.

Discussion

TFP, daunorubicin, and daunorubicinol bind to CSQ with micromolar affinities, and TFP has slightly higher binding affinity than daunorubicin or daunorubicinol. These results are consistent with those obtained using isothermal titration calorimetry, which demonstrated that TFP and daunorubicin are among a number of small molecules that bind to CSQ (Park et al., 2005). In contrast to those results, our data indicate that drug binding affinity is not continually decreased as Ca^{2+} concentration increases. For all three drugs, Ca^{2+} lowered f_1, the fraction of total protein fluorescence quenched by the drug. However, the f_1 decrease (relative to no Ca^{2+}) was similar for 1 and 2.2 mM Ca^{2+}. Ca^{2+} binding alters the effect of the drug on the environment of the CSQ tryptophans. Either the drug occupies different sites on the Ca^{2+}-free versus Ca^{2+}-bound protein or Ca^{2+}-dependent changes in the protein structure (Slupsky et al., 1987; Mitchell et al., 1988; Park et al., 2003) decrease the quenching-sensitivity of the tryptophans. Because higher Ca^{2+} concentration is expected to induce higher order aggregation (Park et al., 2003), the results suggest that the drug binding site(s) is not altered by aggregation or that the tryptophan fluorescence becomes insensitive to changes in drug binding.

The drug binding site(s) on CSQ has not been experimentally identified. However, CSQ contains three thioredoxin-like folds proposed to be the binding site(s) for small hydrophobic ligands, including TFP and daunorubicin (Park et al., 2005). TFP binding has been proposed to inhibit CSQ function by altering the intermolecular interaction between the serine acidic hydrophobic site in domain II and the dibasic hydrophobic site in domain I of CSQ. This contact is observed in the CSQ crystal structure and is thought to be important for protein aggregation and calcium binding (He et al., 1993; Wang et al., 1998). Although TFP binds CSQ with somewhat higher affinity than daunorubicin or daunorubicinol, all three drugs have micromolar apparent binding constants with similar effects on f_1 values. To gain insight into the binding of these drugs to CSQ, we have conducted structural optimization calculations of TFP and daunorubicin. Results show (Fig. 7) that the two drugs are similar in size and shape. The structures differ most in the region of the daunosamine side chain of daunorubicin and the piperazine side chain of TFP. The surface characteristics are also very similar (Fig. 8); the ring structures are mostly neutral in character, whereas the side chain structures are more hydrophilic. These results support the idea that TFP and daunorubicin could bind to the same hydrophobic binding site(s) on CSQ (Park et al., 2005). The difference in binding affinities to CSQ could be conferred by different interactions of the respective side chains with CSQ. Future studies will be required to elucidate the details of the CSQ-drug binding interactions.

Because TFP binding is known to alter CSQ function (He et al., 1993), TFP was chosen to serve as a probe of the effect of CSQ inhibition on SR and cardiac function and to allow for a comparison with the effects of anthracyclines. Caffeine-induced SR Ca^{2+} release from vesicles preincubated with TFP exhibited a concentration-dependent inhibition of the release rate (IC_{50} = 40 μM), which was dependent on the extent of

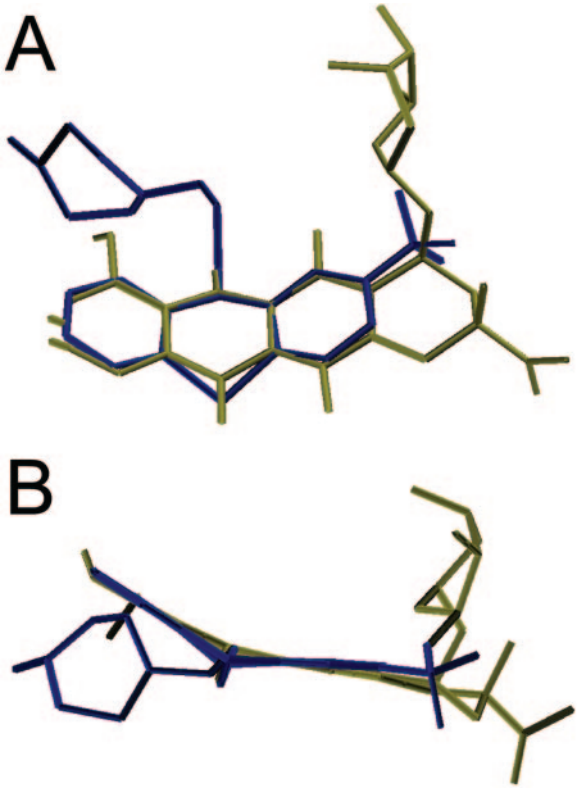

Fig. 7. Overlay of energy-minimized structures of TFP (blue) and daunorubicin (gold) as viewed from the back side (A) and from the bottom (B). Structures were calculated using geometry optimizations completed in Gaussian 98 basis set HF 6-31G*. Representations were generated by the program O (Jones et al., 1991).

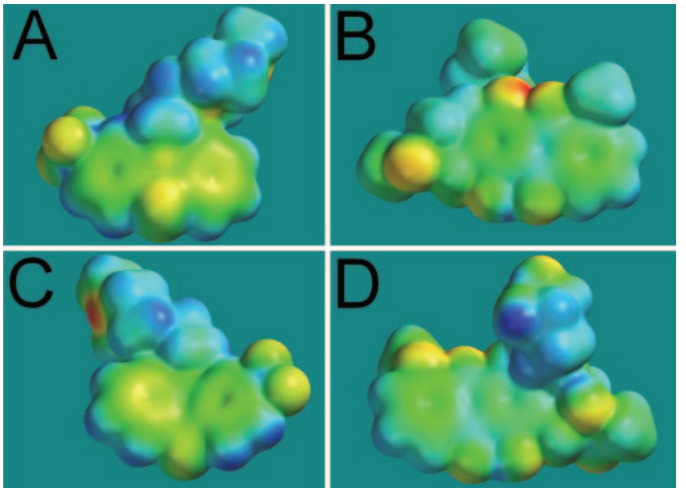

Fig. 8. Representations of the volume-filled, isodensity-mapped surface of TFP (A and C) and daunorubicin (B and D), generated with Spartan 2.0. Views of the front (A and B) and the back (C and D) of each molecule are presented. Structures were calculated as described in Fig. 2. Blue surfaces indicate positive surface density, red surfaces indicate negative surface density, and green indicates neutral surface.

SR Ca^{2+} loading. The earlier TFP was added during the SR Ca^{2+} loading process, the greater the inhibition of release. Inhibition of CSQ function also depends on the order in which Ca^{2+} and TFP are added to the CSQ (He et al., 1993). TFP added to CSQ before Ca^{2+} inhibited CSQ/Ca^{2+} binding, protein conformational changes, and aggregation, whereas addition of TFP after Ca^{2+} had minimal effects (He et al., 1993). Results presented herein, then, are consistent with an inhibitory mechanism involving CSQ as a target. Before Ca^{2+} loading, TFP interacts with CSQ in a manner that disrupts protein function, causing inhibition of Ca^{2+} release. The disruption of CSQ function might involve changes to Ca^{2+} binding, protein aggregation, or its interaction with triadin, junctin, and RyR2. However, if Ca^{2+} is loaded into the SR before the TFP is introduced, then the ability of the drug-CSQ interaction to disrupt CSQ function decreases, and there is less inhibition of Ca^{2+} release. Although it is possible that the inhibition of SR Ca^{2+} release by TFP involves a direct inhibition of the RyR2 Ca^{2+} release channel, TFP has been shown to stimulate, not inhibit, SR Ca^{2+} release in a mechanism similar to that caffeine or micromolar anthracyclines (Wykovsky et al., 1988). Nonetheless, alternate inhibitory mechanisms, other than one that is CSQ-mediated, cannot be ruled out experimentally.

Because inhibition of SR Ca^{2+} release would be expected to alter SR-dependent cardiac contractility, the effects of TFP on contractility (dF/dt) at high rates of contraction and after a rest interval were also explored. The increased strength of contraction at high rates of stimulation and after a rest interval is highly dependent on SR Ca^{2+}. Both conditions increase the SR Ca^{2+} pool (Bouchard and Bose, 1989). Ryanodine and caffeine, which deplete SR Ca^{2+}, effectively antagonize rest-potentiated contractions without altering steady-state contractions (Bers, 1985). In contrast, inhibition of trans-sarcolemmal Ca^{2+} fluxes, as occurs with cobalt and lanthanum, diminish steady-state contractions without altering rested contractions (Bers, 1985; Sutko et al., 1986). Thus, agents that decrease SR Ca^{2+} can selectively impair both rested and high rate-dependent contractions (Burke et al., 2002). The effects of TFP on contractility at high rates of contraction and on rested-contractions suggest that TFP impairs SR Ca^{2+} regulation, possibly by interacting with CSQ, rather than effecting trans-sarcolemmal Ca^{2+} efflux.

It is unlikely that the effects of TFP on cardiac contractility involve the Na^+/Ca^{2+} exchanger. TFP has been reported to inhibit the Na^+/Ca^{2+} exchanger in guinea pig ventricular myocytes (K_d = 7 μM; Kimura, 1993). Inhibition of Na^+/Ca^{2+} exchanger leads to increased SR Ca^{2+} and enhanced contractility of rest-potentiated and high-frequency contractions (Sutko et al., 1986), the opposite of effects of TFP observed in this study. Furthermore, although TFP is a calmodulin inhibitor, calmodulin does not seem to be involved in the observed effects of TFP on either SR Ca^{2+} release or cardiac function. In previous studies, calmodulin-modulated SR Ca^{2+} release rates were insensitive to TFP (Ikemoto et al., 1996), suggesting the effects of TFP on Ca^{2+} release presented here are independent of calmodulin antagonism. Furthermore, inhibition of calmodulin-dependent phosphorylation of phospholamban (Brixius et al., 2003) by TFP, which would enhance SR Ca^{2+} loading and contractile function, would not be expected to produce frequency-dependent changes. Finally, in a study using a series of calmodulin inhibitors in guinea pig papillary muscle preparations, the inhibition of cardiac contractile function by TFP was inconsistent with inhibition of calmodulin by TFP (Koyama and Himori, 1988).

An important aspect of this study is the comparison of the effects of TFP with those previously observed for anthracyclines (Olson et al., 2000; Shadle et al., 2000). The similarity in the effects on SR Ca^{2+} release and cardiac contractility, together with the CSQ binding results, suggest that daunorubicin, daunorubicinol, and TFP could act by a common mechanism, in which inhibition of CSQ function plays a central role. As has been proposed for the action of TFP, the effects of anthracyclines are consistent with a mechanism in which CSQ interacts with daunorubicin or its metabolite in a manner that disrupts protein function, causing inhibition of Ca^{2+} release and impairment of cardiac contractility. Substantiation of this proposed mechanism will require a thorough understanding of the effects of anthracyclines on CSQ function. Despite TFP having a lower K_d for CSQ binding, inhibition of SR Ca^{2+} release and cardiac contractility is more sensitive to anthracyclines. This is possibly caused by different luminal drug concentrations achieved in these experiments.

TFP, when used clinically for treating schizophrenia, does not pose significant cardiac side effects. It is unlikely that clinical doses of TFP, which result in a plasma concentration of approximately 9 nM (Midha et al., 1984; Janicak et al., 1989), are sufficient to reach concentrations that impair SR Ca^{2+} metabolism. The effects of TFP (reversible EKG distortions of the Q and T wave) are minor and relatively rare. Overdose of TFP does not impair contractile function but manifests as cardiac arrhythmias. Thus, the TFP effects described here are not likely to be clinically relevant.

In contrast to the effects of TFP, anthracyclines are clinically observed to impair contractility. Anthracyclines and their metabolites persist in cardiac tissue (Cusack et al., 1993b; Stewart et al., 1993) at concentrations similar to those used in this study. Thus, it is possible that the parent anthracycline, its metabolite, or both interfere with CSQ function in vivo to alter both SR Ca^{2+} regulation and cardiac function. These proposed effects of anthracyclines on CSQ function could act in concert with chronic mechanisms, such as the down-regulation of ryanodine receptor expression (Arai et al., 1998; Burke et al., 2000), to mediate the clinical cardiotoxicity of anthracyclines.

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