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Inhibition of Calcium-induced Calcium Release from Purified Cardiac Sarcoplasmic Reticulum Vesicles*

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Brian K. Chamberlain‡, Pompeo Volpe§, and Sidney Fleischer

From the Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235

A variety of reagents (local anesthetics, phenothiazines, ruthenium red, ryanodine, dicyclohexylcarbodiimide, R 24571) inhibit Ca^{2+}-induced Ca^{2+} release from purified canine cardiac sarcoplasmic reticulum (SR). Most of these compounds also increase the rate of net Ca^{2+} uptake by cardiac SR while moderately inhibiting Ca^{2+}-dependent ATP hydrolysis, and together these two effects produce increased coupling ratios for ATP-dependent Ca^{2+} transport (Ca^{2+}/ATP ≅ 2) compared to controls (Ca^{2+}/ATP ≅ 1). We conclude that Ca^{2+} efflux normally occurs during net Ca^{2+} uptake by isolated cardiac SR vesicles and that this phenomenon is responsible for the low coupling ratios generally observed for cardiac SR preparations.

Blockers of sarcolemmal Ca^{2+} channels (nitrendipine, diltiazem, methoxyverapamil, dantrolene), at concentrations much greater than those effective for sarcolemmal Ca^{2+} fluxes, do not affect either Ca^{2+} uptake or Ca^{2+} release by cardiac SR. Furthermore, the effects of local anesthetics and phenothiazines on Ca^{2+} release from cardiac SR are different from those previously reported for skeletal muscle SR. These results indicate that the Ca^{2+} release "channels" in cardiac SR are distinct from Ca^{2+} channels present in cardiac sarcolemma or in skeletal muscle SR.

The common hydrophobic nature but structural dissimilarity of various inhibitors of Ca^{2+} release from cardiac SR suggest that in some cases a hydrophobic interaction with the membrane might be involved in blocking Ca^{2+} efflux. On the other hand, half-maximal inhibition by 80 nM ruthenium red is suggestive of a specific ionic interaction with some component of the Ca^{2+} efflux pathway.

Contraction and relaxation of muscle fibers are regulated by the myoplasmic Ca^{2+} level. Contraction is triggered by the release of Ca^{2+} from an intracellular membrane system, the SR.[1] Relaxation occurs when the Ca^{2+} is reaccumulated within the lumen of the SR. Ca^{2+} uptake is mediated by a membrane-bound Ca^{2+} pump protein and much is known about this process (2–5). It is generally accepted that 2 mol of Ca^{2+} are transported inside the SR for each mole of ATP hydrolyzed by the pump protein, even though Ca^{2+}/ATP ratios of less than 1 are frequently measured for cardiac SR preparations (6–10). The mechanism of Ca^{2+} release from SR is less well understood (11, 12).

In the companion paper (13), we described a Ca^{2+}-induced Ca^{2+} release from purified cardiac SR vesicles. This Ca^{2+} release is triggered by submicromolar concentrations of extravesicular Ca^{2+} and inhibited by higher Ca^{2+} concentrations and is thus similar to the Ca^{2+}-induced Ca^{2+} release which has been described for skinned cardiac fibers (14, 15). We concluded that this release is not mediated through the Ca^{2+} pump protein, but proceeds through a Ca^{2+}-responsive efflux pathway or "channel(s)." In this report, we describe the inhibition of this Ca^{2+}-induced Ca^{2+} release by a variety of chemical compounds. Moreover, the cardiac SR Ca^{2+} pumping efficiency (Ca^{2+}/ATP) is increased from about 1 to approximately 2 in the presence of many of these compounds.

EXPERIMENTAL PROCEDURES

Materials—Cardiac SR was isolated from canine ventricles as previously described (16) and stored in liquid N_2. Norit A (decolorizing carbon) was from Fisher. Ruthenium red, procaine-HCl, trifluoperazine, and chlorpromazine were obtained from Sigma. [γ-^{32}P]ATP was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). Other drugs were obtained from the following sources: SKF 525-A, Smith Kline and French Laboratories (Philadelphia, PA); ryanodine, S.P. Penick Corp. (Lyndhurst, NJ); R 24571, Janssen Pharmaceutica (Beerse, Belgium); DCCD, Eastman; D600, Knoll Pharmaceutical (Whippany, NJ); diltiazem, Marion Laboratories (Kansas City, MO); nitrendipine, Miles Laboratories (West Haven, CT); dantrolene, Norwich-Eaton Pharmaceuticals (Norwich, NY). The structures of the drugs used in this study are presented in Fig. 1. All other reagents were as described in the preceding report (13).

Trifluoperazine, DCCD, D600, dantrolene, and nitrendipine stock solutions were prepared in ethanol such that the final ethanol concentration in the assay medium was 1% or less. R 24571 was dissolved in Me_2SO such that the Me_2SO concentration in the assay was 0.5% or less. These levels of ethanol and Me_2SO have no effect on either Ca^{2+} uptake or Ca^{2+} release. DCCD and dantrolene solutions were used within 5 h of preparation. SKF 525-A, ruthenium red, procaine, chlorpromazine, ryanodine, and diltiazem stock solutions were prepared in H_2O. Trifluoperazine, chlorpromazine, and nitrendipine were protected from light.

Assays—Enzymic assays were as previously described (13). Net Ca^{2+} uptake and release (in the presence of 1 mM ATP, 75 mM P_i, and an ATP-regenerating system) were observed at 37 °C by dual wavelength spectrophotometry using the metallochromic indicator antipyrylazo III. Ca^{2+} loading by control and SKF 525-A-treated samples were also assayed by using ^{45}Ca and a Millipore filtration technique. Ca^{2+} efflux from passively loaded SR vesicles (in the absence of ATP and P_i) was measured at 25 °C. Briefly, SR vesicles were loaded with $^{45}CaCl_2$ by incubation for 18 h at 0 °C. Efflux was initiated by a 91-fold dilution into warm medium containing 1 μM

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‡ Present address, Calcium Regulation Department, Norwich Eaton Pharmaceuticals, Inc., Norwich, NY 13815.

§ Postdoctoral Fellow of the Muscular Dystrophy Association of America. Present address, Department of Physiology, New Jersey Medical School, 100 Bergen Street, Newark, NJ 07103.

[1] The abbreviations used are: SR, sarcoplasmic reticulum; SKF 525-A, β-diethylaminoethyl diphenylpropylacetate-HCl; R 24571, 1-[bis(p-chlorophenyl)methyl]-3-[2,4,-dichloro-β(2,4-dichlorobenzyloxy)phenethyl]imidazolium chloride; DCCD, dicyclohexylcarbodiimide; D600, methoxyverapamil; Me_2SO, dimethyl sulfoxide; IC_{50}, concentration producing 50% inhibition.

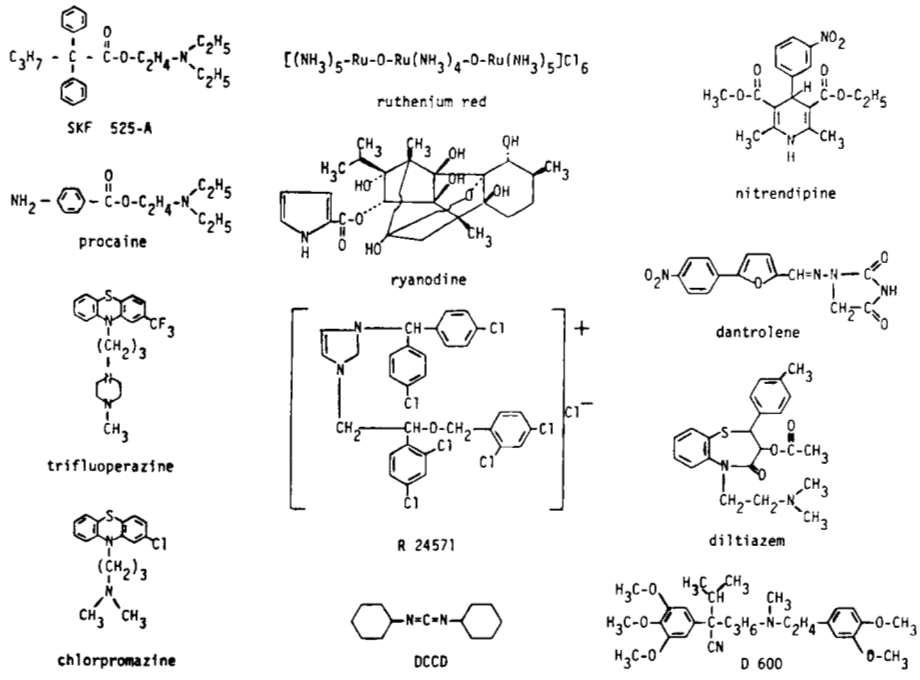

FIG. 1. **Structures of compounds used in this study.** Compounds in the first two columns inhibit Ca^{2+}-induced Ca^{2+} release from isolated cardiac SR vesicles. The sarcolemmal Ca^{2+} channel blockers in the third column have no effect (at the concentrations tested) upon this process.

ionized Ca^{2+} and the drug to be tested. At intervals, samples were removed and filtered. The first order rate constant of the rapid Ca^{2+} efflux (first 35 s) was determined by linear regression analysis of a semilog plot of ^{45}Ca counts/min trapped on the filters *versus* time.

ATPase values at 37 °C were determined spectrophotometrically or by measuring production of $^{32}P_i$ in assays containing [γ-^{32}P]ATP (2000 cpm/nmol). For this procedure, 100-μl aliquots were withdrawn from the standard uptake/release assay medium (13) at 9-s intervals and rapidly mixed with 500 μl of ice-cold 0.1 M H_3PO_4 containing 25 mg of Norit A/ml to adsorb nucleotides. After centrifugation at 1800 $\times$ *g* for 10 min at 4 °C, radioactivity remaining in the supernatants was determined by scintillation counting and the rate of $^{32}P_i$ production was determined by linear regression analysis. All ATPase determinations in the presence of trifluoperazine, chlorpromazine, and R 24571 were done isotopically. ATPase values for the control and in the presence of SKF 525-A were determined by both the spectrophotometric and isotopic procedures. "Basal" values determined in 4 mM EGTA (0.11–0.13 μmol of ATP/mg of protein/min for control samples; less in the presence of some drugs) were subtracted from total values to yield Ca^{2+}-dependent ATPase. "Leaky vesicle ATPase" was determined in the presence of 0.07 mg of Triton X-100/ml. Basal ATPase is reduced and Ca^{2+}-dependent ATP hydrolysis is approximately doubled in the presence of the detergent. This assay is a more accurate reflection of enzyme turnover, unencumbered by a tight Ca^{2+} permeability barrier.

To estimate endogenous calmodulin levels, cardiac SR (300 μl) was heated at 90 °C for 25 min and then sedimented at 1800 $\times$ *g* for 15 min. The supernatant was withdrawn and added to an erythrocyte Ca^{2+}-dependent ATPase assay (17). Stimulation of activity by cardiac SR extracts was compared with that obtained by various concentrations of authentic calmodulin purified from bovine brain (18).

RESULTS

Isolated vesicles of canine cardiac SR exhibit Ca^{2+}-induced Ca^{2+} release (13). A typical assay is illustrated in Fig. 2*A*, where the SR was preloaded with three additions of $CaCl_2$. Following a fourth addition of $CaCl_2$ to the control sample (*trace 1*), net Ca^{2+} uptake ceases and net Ca^{2+} release begins. The rate and extent of Ca^{2+} release depend on the extravesicular ionized Ca^{2+} concentration after the SR vesicles accumulate Ca^{2+} to a trigger capacity (13). Maximum Ca^{2+} release occurs at 1–2 μM extravesicular Ca^{2+}. After 2–3 min of Ca^{2+} release, Ca^{2+} reuptake by the SR occurs.

When the local anesthetic SKF 525-A is present throughout

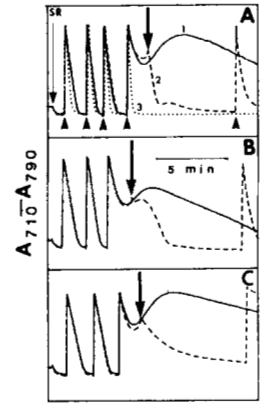

FIG. 2. **Inhibition of Ca^{2+}-induced Ca^{2+} release.** Net Ca^{2+} uptake and release were determined spectrophotometrically, the absorbance being proportional to the extravesicular ionized Ca^{2+} concentration. Thus, net Ca^{2+} uptake by the SR is accompanied by a *decrease* in absorbance and net Ca^{2+} release is reflected by an increase in absorbance. *A*, at the *small arrow*, SR (40 μg of protein) was added to the medium and allowed to accumulate contaminating Ca^{2+} (approximately 4 nmol) prior to four additions of 43 nmol of $CaCl_2$ (*arrowheads*) in a total volume of 1 ml. In the control *trace 1* (*solid line*), net Ca^{2+} uptake ceased before all the Ca^{2+} was accumulated by the SR and a period of net Ca^{2+} release ensued. After 2–3 min of release, a slow Ca^{2+} reuptake by the SR occurred. In *trace 2* (*dashed line*), 120 μM SKF 525-A was added when Ca^{2+} release was beginning (*large arrow*). Later, another 43 nmol of $CaCl_2$ (*arrowhead*) was added to recalibrate the dye in the presence of the drug. In *trace 3* (*dotted line*), 120 μM SKF 525-A was already present in the medium at the time of SR addition. The same experimental protocol was used for *B* and *C* below with different SR preparations and different drugs. *B*, following three additions of 45 nmol of $CaCl_2$ to 41 μg of SR, the control sample (*solid line*) exhibited Ca^{2+} release/reuptake. When 400 μM ryanodine was added at the start of release (*arrow*), Ca^{2+} release was inhibited (*dashed line*) and the SR could accumulate the 45 nmol of $CaCl_2$ added to recalibrate the dye. *C*, following three additions of 40 nmol of $CaCl_2$ to 38 μg of SR, the control sample (*solid line*) exhibited Ca^{2+} release/reuptake. When 180 μM DCCD was added at the start of release (*arrow*), Ca^{2+} release was inhibited (*dashed line*), but some 5–10 min later, the SR could not accumulate the 40 nmol of $CaCl_2$ added to recalibrate the dye.

the Ca^{2+} preloading of the SR, the fourth addition of $CaCl_2$ does not elicit Ca^{2+} release (Fig. 2*A*, *trace 3*). Moreover, the rate of Ca^{2+} loading in the presence of the anesthetic is increased almost 2-fold relative to the control. The addition of SKF 525-A to the SR just as Ca^{2+} release begins stops the release *immediately* and rapid Ca^{2+} uptake ensues (Fig. 2*A*, *trace 2*). In the presence of the anesthetic, the SR accumulates additional Ca^{2+} without net Ca^{2+} release.

A wide variety of chemical compounds including ruthenium red, trifluoperazine, chlorpromazine, ryanodine, procaine, R 24571 (a calmodulin antagonist, see Ref. 19), and DCCD exert similar influences on Ca^{2+} uptake and release by cardiac SR (see Fig. 1 for structures). Each of these compounds, with the exception of ryanodine, is immediately effective in blocking Ca^{2+}-induced Ca^{2+} release from cardiac SR, as shown for SKF 525-A in Fig. 2*A* (*trace 2*). When ryanodine is added at the onset of Ca^{2+} release, net release proceeds at a slower rate for approximately 40 s before maximum inhibition of release and rapid Ca^{2+} uptake occurs (Fig. 2*B*). Even DCCD, at a concentration which significantly inhibits the turnover of the Ca^{2+}-dependent ATPase (Table I), produces immediate Ca^{2+} uptake when added during Ca^{2+} release (Fig. 2*C*). In contrast to compounds less inhibitory for the ATPase (Fig. 2, *A* and *B*), after 5–10 min in the presence of DCCD, the SR accumulates additional Ca^{2+} very slowly (Fig. 2*C*).

As shown for SKF 525-A in Fig. 2*A* (*trace 3*), most of these compounds stimulate the rate of net Ca^{2+} uptake when they are present prior to Ca^{2+} loading of the SR. The effect on Ca^{2+} loading does not appear to be dependent on the extravesicular Ca^{2+} concentration since linear rates of uptake are observed over a range of external Ca^{2+} both in the presence and absence of inhibitors (*e.g.* see Fig. 2*A*). Enhanced Ca^{2+} loading occurs even though Ca^{2+}-dependent ATP hydrolysis is reduced (Table I). The net result is an increase in the coupling ratios (Ca^{2+}/ATP) for ATP-dependent Ca^{2+} transport. For SKF 525-A, these findings were confirmed by measuring Ca^{2+} loading and ATP hydrolysis by spectrophotometric and isotopic methods (see "Experimental Procedures"). Some compounds which significantly inhibit the turnover of the Ca^{2+}-dependent ATPase enzyme such as R 24571 and DCCD (Table I) fail to increase the rate of Ca^{2+} loading. But, only DCCD, the most effective inhibitor of Ca^{2+}-dependent ATPase (at the concentrations used in this study), fails to increase the Ca^{2+} pumping efficiency. The other compounds listed in Table I are more effective inhibitors of Ca^{2+} efflux than they are of Ca^{2+} pump turnover and therefore increase Ca^{2+}/ATP ratios by blocking Ca^{2+} efflux which normally occurs during net Ca^{2+} uptake. In the presence of most Ca^{2+} efflux inhibitors, the Ca^{2+}/ATP ratios are increased from approximately 1 to about 2.

Representative of four classes of sarcolemmal Ca^{2+} channel blockers were also tested. None of these compounds (2 μM nitrendipine, 10 μM diltiazem, 20 μM dantrolene, or 75 μM D600, see Fig. 1 for structures) has any effect on Ca^{2+} uptake or release by cardiac SR vesicles. In the presence of these compounds, Ca^{2+} uptake parallels control samples and the rate and extent of Ca^{2+}-induced Ca^{2+} release are likewise indistinguishable from control samples (data not shown).

Compounds which inhibit Ca^{2+} release from actively loaded SR also inhibit Ca^{2+} efflux from passively loaded SR vesicles (Table II). The rate constants for efflux in the presence of SKF 525-A, ruthenium red, trifluoperazine, and DCCD are similar to that previously observed for Ca^{2+}-*independent* Ca^{2+} efflux (13). It appears that the same concentrations of these compounds which are maximally effective in inhibiting Ca^{2+}-induced Ca^{2+} release from actively loaded SR (see legend to

TABLE I

Effects of inhibitors of Ca^{2+} release on Ca^{2+} loading and Ca^{2+}-dependent ATPase

Addition[a]	Ca^{2+} loading[b]	Ca^{2+}-ATPase[c]	Ca^{2+}/ATP	"Leaky vesicle Ca^{2+}-ATPase"[d]	
	μmol Ca^{2+}/mg protein/min	*μmol ATP/mg protein/min*		*μmol/mg protein/min*	%
None	0.96	1.16	0.8	1.84	100
SKF 525-A, 120 μM	2.02 (9)	0.94 (5)	2.1	1.53 (3)	83.2
Trifluoperazine, 40 μM	1.84 (7)	0.87 (2)	2.1	1.31 (2)	71.2
Ruthenium red, 12 μM	1.65 (8)	0.82 (3)	2.0	1.71 (2)	92.9
Chlorpromazine, 160 μM	1.35 (5)	0.63 (2)	2.1	1.00 (1)	54.3
Procaine, 8 mM	1.12 (6)	0.98 (3)	1.1	1.68 (2)	91.3
R 24571, 20 μM	0.99 (7)	0.70 (1)	1.4	1.21 (1)	65.8
None	1.01	0.90	1.1	2.10	100
Ryanodine, 400 μM	1.33 (5)	0.70 (2)	1.9	1.90 (1)	90.5
DCCD, 180 μM	0.63 (3)	0.57 (1)	1.1	0.72 (1)	34.3

[a] Drug concentrations are the minimum which are *maximally effective* both in inhibiting net Ca^{2+} release when added at the onset of release and in enhancing Ca^{2+} uptake when present before addition of $CaCl_2$ (see Fig. 2). Similar drug effects were observed with six SR preparations. Since the control Ca^{2+} loading (0.92–1.35 μmol/mg/min), Ca^{2+}-ATPase (0.90–1.18 μmol/mg/min), and Ca^{2+}/ATP (0.8–1.2) varied among the preparations, data are presented only from typical experiments using two different SR preparations.

[b] Ca^{2+} loading was determined in the same medium as was Ca^{2+}-dependent ATPase so that accurate coupling ratios could be calculated. When the spectrophotometric ATPase assay was used (see "Experimental Procedures"), the modified uptake medium containing the alternate ATP regenerating system described in the preceding report (13) was employed for both loading and ATPase determinations. The *numbers* in *parentheses* indicate the number of experiments in which a similar effect on Ca^{2+} loading by various SR preparations was consistently observed.

[c] Ca^{2+}-dependent ATPase values were determined either spectrophotometrically or isotopically as described under "Experimental Procedures." The *numbers* in *parentheses* indicate the number of paired loading/ATPase experiments in which similar effects on Ca^{2+}-ATPase and Ca^{2+}/ATP values for various SR preparations were consistently observed.

[d] Leaky vesicle Ca^{2+}-ATPase was determined as described under "Experimental Procedures" to determine the effects of the drugs on enzyme turnover. Under the conditions of this assay, the "basal" ATPase is typically less than 1% of the Ca^{2+}-dependent ATPase, so a separate basal ATPase determination was not performed. The *numbers* in *parentheses* indicate the number of experiments in which similar effects on the leaky vesicle Ca^{2+}-ATPase activity of various SR preparations were observed.

TABLE II

Effects of drugs on Ca^{2+} efflux from passively loaded cardiac SR vesicles

Ca^{2+} efflux at 1 μM extravesicular ionized Ca^{2+} from passively loaded vesicles was determined as described under "Experimental Procedures." This Ca^{2+} concentration is optimal for Ca^{2+}-induced Ca^{2+} efflux from passively loaded SR (13). Addition of drugs was coincident with dilution of the SR to initiate efflux. The data are the first order rate constants for the rapid initial phase of Ca^{2+} efflux (first 35 s). Values are for a single experiment except for the control and ryanodine data which are the mean ± S.D. from two experiments.

Addition[a]	k[b]
	min^{-1}
None	1.30 ± 0.00
DCCD, 180 μM	0.34
Ruthenium red, 12 μM	0.35
SKF 525-A, 120 μM	0.36
Trifluoperazine, 40 μM	0.41
Ryanodine, 400 μM	0.74 ± 0.07
Diltiazem, 10 μM	1.31
Calmodulin, 1 μM	1.21

[a] Drug concentrations are the minimum which are maximally effective in inhibiting Ca^{2+} release from actively loaded SR vesicles.

[b] The first order rate constant for the rapid initial phase of Ca^{2+} efflux for a control sample at 1 nM extravesicular ionized Ca^{2+} (*i.e.* Ca^{2+}-*independent* efflux) was 0.35 (13).

TABLE III

Potency of inhibitors of Ca^{2+} release

Compounds which inhibit Ca^{2+}-induced Ca^{2+} release from cardiac SR were tested for their potency in enhancing the rate of Ca^{2+} loading. Various concentrations of each drug were added to the standard uptake/release medium (13) prior to addition of SR (40 μg of protein). Ca^{2+} uptake in a final volume of 1 ml was initiated by addition of $CaCl_2$. The loading rate (micromoles of Ca^{2+}/mg of protein/min) of control samples without drugs was subtracted from loading rates in the presence of drugs to determine the level of loading stimulation. For each drug, 5–10 different concentrations were evaluated in each of two separate experiments and a composite curve was constructed to relate loading stimulation to drug concentration. Values presented are the drug concentrations which gave half-maximal stimulation under the assay conditions.

Drug	IC_{50}
	μM
Ruthenium red	0.08
Trifluoperazine	28
Chlorpromazine	53
SKF 525-A	65
Ryanodine	130
Procaine	2100

Table I) can completely block the Ca^{2+}-dependent component of Ca^{2+} efflux from passively loaded vesicles. On the other hand, the optimum concentration of ryanodine does not reduce Ca^{2+} efflux from passively loaded SR to the same extent as do the other compounds. Since the determination of the rate constant for efflux from passively loaded SR includes data only from the first 35 s of efflux, this result is consistent with the observation that the maximum effect of ryanodine after ATP-dependent Ca^{2+} loading is not observed until about 40 s after addition of the drug (Fig. 2*B*). Also consistent with results from actively loaded SR, a sarcolemmal Ca^{2+} channel blocker such as diltiazem has no effect on Ca^{2+} efflux from passively loaded SR (Table II).

The data presented thus far have been for drugs at their maximum effective concentrations, but these concentrations do not indicate the relative potency of the Ca^{2+} release inhibitors. The concentrations which produce 50% stimulation of Ca^{2+} loading are given in Table III. We assume that enhanced Ca^{2+} loading in the presence of the drugs reflects their efficacy in blocking unidirectional Ca^{2+} efflux which otherwise occurs during net Ca^{2+} uptake. A similar explanation was previously suggested regarding the effects of ryanodine and ruthenium red on Ca^{2+} uptake by canine cardiac SR (20, 21). The data are presented in terms of IC_{50} rather than as inhibition constants since most of the compounds tested are hydrophobic and bind to membranes and glass. It is therefore difficult to determine the actual drug concentration in solution (*e.g.* see discussions in Refs. 22 and 23 regarding trifluoperazine), so that the effectiveness of a drug can depend upon assay conditions, especially membrane concentration (*e.g.* see Refs. 24 and 25 regarding trifluoperazine). Under the conditions of our assay, ruthenium red (IC_{50} = 80 nM) is the most potent inhibitor of Ca^{2+} efflux and procaine is the least potent.

Since several of the compounds—trifluoperazine (26–28), chlorpromazine (26–28), R 24571 (19), and SKF 525-A (29)—which inhibit Ca^{2+} release from cardiac SR are calmodulin antagonists, we investigated whether calmodulin might be involved in Ca^{2+} efflux. The endogenous calmodulin level in the SR was estimated to be 7.3 pmol/mg of protein, or about 0.3 nM in the standard uptake/release assay containing 40 μg of protein/ml. If these drugs were to inhibit Ca^{2+} release by interacting with endogenous calmodulin on the SR vesicles, addition of exogenous calmodulin might be expected to promote Ca^{2+} release and/or prevent the inhibition of release by the drugs. However, Ca^{2+} efflux from passively loaded SR vesicles is not stimulated by 1 μM exogenous calmodulin (Table II) and addition of 1.5 μM exogenous calmodulin at the onset of Ca^{2+}-induced Ca^{2+} release from actively loaded SR (at 3 μM extravesicular ionized Ca^{2+}) has no effect on the rate or extent of release (data not shown), although reuptake of the released Ca^{2+} is faster in the presence of exogenous calmodulin, presumably due to enhanced Ca^{2+} transport activity following calmodulin-dependent phosphorylation of the cardiac SR (4, 16, 30, 31). Moreover, when 4.5 μM calmodulin (15,000 times the endogenous level) is added at the onset of Ca^{2+} release, the subsequent addition of 40 μM trifluoperazine is still maximally effective in immediately inhibiting Ca^{2+} release and producing rapid Ca^{2+} accumulation. Thus, we were unable to directly demonstrate an involvement of calmodulin in Ca^{2+} efflux.

DISCUSSION

We find that compounds of diverse structure—the local anesthetics SKF 525-A and procaine, the phenothiazines trifluoperazine and chlorpromazine, ruthenium red, ryanodine, DCCD, and the calmodulin antagonist R 24571—inhibit Ca^{2+} efflux from purified cardiac SR vesicles. With the exception of ryanodine which requires approximately 40 s for maximum effectiveness, these compounds *immediately* block both net Ca^{2+}-induced Ca^{2+} release from actively loaded SR and the Ca^{2+}-dependent component of Ca^{2+} efflux from passively loaded SR. This report is the first demonstration that local anesthetics, phenothiazines, DCCD, and R 24571 can block Ca^{2+} efflux from cardiac SR. Also, even though ryanodine and ruthenium red have previously been indirectly implicated in the inhibition of Ca^{2+} release from cardiac SR (20, 21, 67), this report provides direct evidence of the effects of these drugs on Ca^{2+} efflux from cardiac SR.

The use of Ca^{2+} efflux inhibitors enables us to make several conclusions regarding Ca^{2+} pumping *into* cardiac SR. Although it is generally accepted that 2 mol of Ca^{2+} are transported inside the SR for each mole of ATP hydrolyzed by the Ca^{2+} pump protein (2), Ca^{2+}/ATP ratios of less than 1 are frequently measured for cardiac SR (6–10). Ca^{2+} efflux inhibitors increase the rate of Ca^{2+} loading by cardiac SR while

having a moderate inhibitory effect upon Ca^{2+}-dependent ATP hydrolysis. These two effects together produce increased coupling ratios for ATP-dependent Ca^{2+} transport. Of the inhibitors described in this report, only DCCD (which is also the most potent inhibitor of Ca^{2+}-dependent ATPase at the concentrations tested) fails to increase Ca^{2+}/ATP. Since measurements of net Ca^{2+} uptake reflect the differences between unidirectional Ca^{2+} influx and efflux rates, we conclude that in the absence of inhibitors, considerable Ca^{2+} efflux occurs during net Ca^{2+} uptake by most preparations of cardiac SR and that this phenomenon is responsible for the low coupling ratios generally observed. Under the assay conditions of this study, Ca^{2+}/ATP ratios of approximately 2 can be achieved by loading cardiac SR in the presence of SKF 525-A, trifluoperazine, ruthenium red, chlorpromazine, or ryanodine which block Ca^{2+} efflux so that unidirectional Ca^{2+} influx is determined. In the case of SKF 525-A, for example, measured Ca^{2+} loading rates are approximately doubled in the presence of the drug compared to control even though Ca^{2+}-ATPase turnover is reduced.

The stimulation of Ca^{2+} loading cannot be ascribed to artifacts of the assay procedure. Similar results were obtained for SKF 525-A when Ca^{2+} loading and ATPase were determined either by spectrophotometric methods or by isotopic procedures (see "Experimental Procedures"). Moreover, similar concentrations of SKF 525-A and procaine (32) and trifluoperazine and chlorpromazine (91) were found to *inhibit* Ca^{2+} loading by skeletal muscle SR when assayed by the same procedure in virtually identical uptake/release medium. The results can, however, be influenced by the assay medium. When cardiac SR is assayed in a $CaCl_2$/EGTA buffered medium using high concentrations of MgATP and oxalate as a precipitating anion (16), Ca^{2+} loading and ATPase rates for control samples of the same SR preparations are both about 2.5-fold faster than those in Table I. Under these conditions, addition of SKF 525-A increases the loading rate only about 20% and Ca^{2+}/ATP increases to only 1.5 (data not shown). Differences in SR preparation or experimental protocol may explain why inhibition of Ca^{2+} loading was observed with R 24571 in a previous report (33), whereas in this study no inhibition was observed (Table I).

Since Ca^{2+} loading is stimulated by the same compounds which inhibit Ca^{2+}-dependent Ca^{2+} efflux from both actively and passively loaded SR, we conclude that the Ca^{2+} efflux which occurs during net Ca^{2+} uptake proceeds through the same or similar channels as does the Ca^{2+}-triggered Ca^{2+} efflux. Conceivably, some channels might lose their Ca^{2+} gating property during SR vesicle isolation, by a conformational rearrangement of the channel itself or through loss of a regulatory factor. Different degrees of damage during different isolation procedures could leave more or fewer channels "open." This variation would be additive to that resulting from different proportions of the SR subpopulation possessing the presumptive channels (9, 21). Variability in the number of open Ca^{2+} channels could explain some of the diversity in Ca^{2+} loading specific activity and in coupling ratios observed for preparations of similar Ca^{2+} pump protein content and apparent purity.

Although several of the Ca^{2+} efflux inhibitors—trifluoperazine, chlorpromazine, R 24571, and SKF 525-A—can act as calmodulin antagonists (19, 26–29), we cannot conclude from our data that Ca^{2+} efflux from cardiac SR involves calmodulin. Exogenous calmodulin, at several thousand-fold excess over the endogenous level, has no effect on Ca^{2+}-induced Ca^{2+} release or on the inhibition of Ca^{2+} release by trifluoperazine. Moreover, inhibition of calmodulin-*independent* activities by calmodulin antagonists is well documented (33–41).

It is conceivable that the calmodulin antagonists might interact with a "calmodulin-like" protein tightly associated with the Ca^{2+} efflux channel. Since the channel gating is sensitive to Ca^{2+}, some portion of the channel could be a Ca^{2+}-binding protein. For example, calmodulin antagonists apparently bind to receptor sites for sarcolemmal Ca^{2+} channel blockers (42, 43) and certain sarcolemmal Ca^{2+} channel blockers bind calmodulin (43–45), leading to the speculation that a calmodulin-like site is present on sarcolemmal Ca^{2+} channels (42, 43,46). A similar site might exist on cardiac SR Ca^{2+} channels.

On the other hand, inhibition of Ca^{2+} efflux by calmodulin antagonists, and other compounds as well, could involve their previously documented hydrophobicity and interactions with phospholipids (36, 37, 41, 47–55). The importance of hydrophobicity in otherwise similar reagents can be visualized by comparing the structure of the potent Ca^{2+} efflux inhibitor SKF 525-A with that of the much less potent and less hydrophobic procaine (Fig. 1). However, it is doubtful that compounds such as SKF 525-A affect Ca^{2+} efflux by altering the general physical properties of the SR membrane phospholipids, since a larger effect on Ca^{2+}-dependent ATPase would be expected in that case. Also, the biochemical effects of phenothiazines have been correlated with their ability to stabilize membranes (51), but it is doubtful that this property is responsible for the inhibition of Ca^{2+} efflux from cardiac SR, since phenothiazines do not reduce Ca^{2+} efflux from skeletal muscle SR under similar conditions (91).

Regardless of the mechanism of action, this study of the inhibition of Ca^{2+} efflux from isolated cardiac SR vesicles provides new information regarding the similarities and differences among the Ca^{2+} "channels" present in cardiac sarcolemma, cardiac SR, and skeletal muscle SR membranes. For example, representatives of four classes of sarcolemmal Ca^{2+} channel blockers have no effect on Ca^{2+} efflux from cardiac SR at concentrations much higher than those effective in blocking sarcolemmal Ca^{2+} channels (56–59). These data are consistent with previous results, obtained with different SR preparations and assay conditions, indicating that similar concentrations of verapamil, D600, dantrolene, and dihydropyridines such as nitrendipine have no effect on either Ca^{2+} loading or release by cardiac SR vesicles (60–65). Nitrendipine has been shown to bind very tightly (dissociation constant = 0.2–0.4 nM) to the same subpopulation of SR vesicles which is sensitive to ryanodine and ruthenium red (21, 66). However, concentrations of nitrendipine 10,000-fold greater than the dissociation constant do not affect Ca^{2+} release. The failure of sarcolemmal Ca^{2+} channel blockers to affect Ca^{2+} efflux from cardiac SR indicates structural diversity for the sarcolemmal and SR Ca^{2+} channels.

The results of this study show some similarities to those obtained with skeletal muscle SR. For example, ruthenium red (68–74), DCCD (71, 75), and local anesthetics such as procaine (11, 72, 74–78) have been found to also block a Ca^{2+}-induced Ca^{2+} release from "heavy" skeletal muscle SR vesicles. In addition, local anesthetics such as procaine and SKF 525-A block a spontaneous Ca^{2+} release from skeletal muscle SR (32) and procaine and DCCD inhibit Ca^{2+} release from skeletal muscle SR induced by others means (79–81). Moreover, ruthenium red can enhance the rate of Ca^{2+} loading by heavy skeletal muscle SR (68, 82) and increase the Ca^{2+}/ATP coupling ratio (82). In these respects, these compounds similarly affect cardiac and skeletal muscle SR.

On the other hand, many drug effects are dissimilar for the two systems. For example, ryanodine has been reported to *stimulate* Ca^{2+} efflux from heavy skeletal muscle SR (83).

Also, chlorpromazine and trifluoperazine stimulate net Ca^{2+} release from skeletal muscle SR (25, 80, 84, 91) and trifluoperazine *decreases* the Ca^{2+}/ATP coupling ratio in skeletal muscle SR (24). The enhanced Ca^{2+} release in the presence of phenothiazines appears to be due to inhibition of the Ca^{2+} pump since the drugs do not affect unidirectional Ca^{2+} efflux from either actively or passively loaded skeletal muscle SR[2] (78, 85). Finally, even though local anesthetics block spontaneous Ca^{2+} release from skeletal muscle SR, they do so in a time-dependent manner (32), whereas the inhibition of Ca^{2+} efflux from cardiac SR by these compounds is immediate.

Our data thus indicate diversity in the Ca^{2+} channels in cardiac SR, cardiac sarcolemma, and skeletal muscle SR membranes. The diversity may be in the structural components of the channels themselves or in some regulatory factors which control the channel gating. In this regard, it should be noted that multiple binding sites for Ca^{2+} channel inhibitors have been suggested for both sarcolemma (86–89) and skeletal muscle SR (90) membranes, and drug binding sites might be different from the actual Ca^{2+} efflux channels. As noted above, the more hydrophobic compounds might block Ca^{2+} efflux by interacting with phospholipids. Of the inhibitors of Ca^{2+} efflux from cardiac SR, ruthenium red is most likely to interact directly with a Ca^{2+} efflux channel, since it is the most potent (IC_{50} = 80 nM) and the least hydrophobic (a hexavalent cation—see Fig. 1). It is equally potent (IC_{50} = 40–70 nM) in blocking Ca^{2+} efflux from the ryanodine-sensitive cardiac SR subpopulation (21) and from heavy skeletal muscle SR (71, 74). These data suggest a specific, nonhydrophobic interaction with a component common to Ca^{2+} efflux channels in both cardiac and skeletal muscle SR membranes.

REFERENCES

1. Chamberlain, B. K., Volpe, P., and Fleischer, S. (1983) *Fed. Proc.* **42,** 2215
2. Tada, M., Yamamoto, T., and Tonomura, Y. (1978) *Physiol. Rev.* **58,** 1–79
3. Møller, J. V., Andersen, J. P., and le Maire, M. (1982) *Mol. Cell. Biochem.* **42,** 83–107
4. Tada, M., Yamada, M., Kadoma, M., Inui, M., and Ohmori, F. (1982) *Mol. Cell. Biochem.* **46,** 73–95
5. Ikemoto, N. (1982) *Annu. Rev. Physiol.* **44,** 297–317
6. Jones, L. R., Besch, H. R., Jr., Sutko, J. L., and Willerson, J. T. (1979) *J. Pharmacol. Exp. Ther.* **209,** 48–55
7. Feher, J. J., and Briggs, F. N. (1980) *Cell Calcium* **1,** 105–118
8. Feher, J. J., Briggs, F. N., and Hess, M. L. (1980) *J. Mol. Cell Cardiol.* **12,** 427–432
9. Jones, L. R., and Cala, S. E. (1981) *J. Biol. Chem.* **256,** 11809–11818
10. Kirchberger, M. A., and Antonetz, T. (1982) *J. Biol. Chem.* **257,** 5685–5691
11. Endo, M. (1977) *Physiol. Rev.* **57,** 71–108
12. Winegrad, S. (1982) *Annu. Rev. Physiol.* **44,** 451–462
13. Chamberlain, B. K., Volpe, P., and Fleischer, S. (1984) *J. Biol. Chem.* **259,** 7540–7546
14. Fabiato, A. (1982) *Fed. Proc.* **41,** 2238–2244
15. Fabiato, A. (1983) *Am. J. Physiol.* **245,** C1–C14
16. Chamberlain, B. K., Levitsky, D. O., and Fleischer, S. (1983) *J. Biol. Chem.* **258,** 6602–6609
17. Gietzen, K., Wüthrich, A., and Bader, H. (1981) *Biochem. Biophys. Res. Commun.* **101,** 418–425
18. Watterson, D. M., Harrelson, W. G., Jr., Keller, P. M., Sharief, F., and Vanaman, T. C. (1976) *J. Biol. Chem.* **251,** 4501–4513
19. Van Belle, H. (1981) *Cell Calcium* **2,** 483–494
20. Jones, L. R., Besch, H. R., Jr., Sutko, J. L., and Willerson, J. T. (1979) *J. Pharmacol. Exp. Ther.* **209,** 48–55
21. Jones, L. R., and Burrows, S. D. (1983) *Fed. Proc.* **42,** 1933
22. Raess, B. U., and Vincenzi, F. F. (1980) *Mol. Pharmacol.* **18,** 253–258
23. Hinds, T. R., Raess, B. U., and Vincenzi, F. F. (1981) *J. Membr. Biol.* **58,** 57–65
24. Campbell, K. P., and MacLennan, D. H. (1982) *J. Biol. Chem.* **257,** 1238–1246
25. Ho, M.-M., Scales, D. J., and Inesi, G. (1983) *Biochim. Biophys. Acta* **730,** 64–70
26. Weiss, B., and Wallace, T. L. (1980) in *Calcium and Cell Function* (Cheung, W. Y., ed) Vol. 1, pp. 329–379, Academic Press, New York
27. Weiss, B., Prozialeck, W., Cimino, M., Barnette, M. S., and Wallace, T. L. (1980) *Ann. N. Y. Acad. Sci.* **356,** 319–345
28. Weiss, B., Prozialeck, W. C., and Wallace, T. L. (1982) *Biochem. Pharmacol.* **31,** 2217–2226
29. Volpi, M., Sha'afi, R. I., Epstein, P. M., Andrenyak, D. M., and Feinstein, M. B. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 795–799
30. Tada, M., and Katz, A. M. (1982) *Annu. Rev. Physiol.* **44,** 401–423
31. Tada, M., and Inui, M. (1983) *J. Mol. Cell. Cardiol.* **15,** 565–575
32. Volpe, P., Palade, P., Costello, B., Mitchell, R. D., and Fleischer, S. (1983) *J. Biol. Chem.* **258,** 12434–12442
33. Louis, C. F., Turnquist, J., and Jarvis, B. (1983) *Cell Calcium* **4,** 107–116
34. Roufogalis, B. D. (1982) in *Calcium and Cell Function* (Cheung, W. Y., ed) Vol. 3, pp. 129–159, Academic Press, New York
35. Roufogalis, B. D. (1975) *J. Neurochem.* **24,** 51–61
36. Mori, T., Takai, Y., Minakuchi, R., Yu, B., and Nishizuka, Y. (1980) *J. Biol. Chem.* **255,** 8378–8380
37. Schatzman, R. C., Wise, B. C., and Kuo, J. F. (1981) *Biochem. Biophys. Res. Commun.* **98,** 669–676
38. Wrenn, R. W., Katoh, N., Schatzman, R. C., and Kuo, J. F. (1981) *Life Sci.* **29,** 725–733
39. Wise, B. C., Raynor, R. L., and Kuo, J. F. (1982) *J. Biol. Chem.* **257,** 8481–8488
40. Wise, B. C., Glass, D. B., Jen Chou, C.-H., Raynor, R. L., Katoh, N., Schatzman, R. C., Turner, R. S., Kibler, R. F., and Kuo, J. F. (1982) *J. Biol. Chem.* **257,** 8489–8495
41. Wise, B. C., and Kuo, J. F. (1983) *Biochem. Pharmacol.* **32,** 1259–1265
42. Kauffman, R. F., and Conery, B. G. (1983) *Fed. Proc.* **42,** 573
43. Thayer, S. A., and Fairhurst, A. S. (1983) *Mol. Pharmacol.* **24,** 6–9
44. Bostrom, S.-L., Ljung, B., Mardh, S., Forsen, S., and Thulin, E. (1981) *Nature (Lond.)* **292,** 777–778
45. Johnson, J. D., and Wittenauer, L. A. (1983) *Biochem. J.* **211,** 473–479
46. Johnson, J. D. (1984) *Biophys. J.* **45,** 134–136
47. Roufogalis, B. D. (1981) *Biochem. Biophys. Res. Commun.* **98,** 607–613
48. Tanaka, T., and Hidaka, H. (1981) *Biochem. Biophys. Res. Commun.* **101,** 447–453
49. Prozialeck, W. C., and Weiss, B. (1982) *J. Pharmacol. Exp. Ther.* **222,** 509–516
50. Vincenzi, F. F. (1981) *Proc. West. Pharmacol. Soc.* **24,** 193–196
51. Landry, Y., Amellal, M., and Ruckstuhl, M. (1981) *Biochem. Pharmacol.* **30,** 2031–2032
52. Papahadjopoulos, D. (1972) *Biochim. Biophys. Acta* **265,** 169–186
53. Neal, M. J., Butler, K. W., Polnaszek, C. F., and Smith, I. C. P. (1976) *Mol. Pharmacol.* **12,** 144–155
54. Frenzel, J., Arnold, K., and Nuhn, P. (1978) *Biochim. Biophys. Acta* **507,** 185–197
55. Pang, K.-Y. Y., and Miller, K. W. (1978) *Biochim. Biophys. Acta* **511,** 1–9
56. Triggle, D. J., and Swamy, V. C. (1980) *Chest* **78,** (suppl.) 174–179
57. Triggle, D. J. (1981) in *New Perspectives on Calcium Antagonists* (Weiss, G. B., ed) pp. 1–18, American Physiological Society, Bethesda, MD
58. Triggle, D. J. (1982) in *Calcium Regulation by Calcium Antagonists* (Rahwan, R. G., and Witiak, D. T., eds) pp. 17–37, American Chemical Society, Washington, D. C.
59. Triggle, D. J., and Swamy, V. C. (1983) *Circ. Res.* **52,** Suppl. I, 17–28
60. Nayler, W. G., and Szeto, J., (1972) *Cardiovasc. Res.* **6,** 120–128
61. Entman, M. L., Allen, J. C., Bornet, E. P., Gillette, P. C., Wallick, E. T., and Schwartz, A. (1972) *J. Mol. Cell. Cardiol.* **4,** 681–687
62. Watanabe, A. M., and Besch, H. R., Jr. (1974) *J. Pharmacol. Exp.*

Ther. **191,** 241–251
63. Van Winkle, W. B. (1976) *Science* **193,** 1130–1131
64. Dunnett, J., and Nayler, W. G. (1978) *J. Mol. Cell. Cardiol.* **10,** 487–498
65. Colvin, R. A., Pearson, N., Messineo, F. C., and Katz, A. M. (1982) *J. Cardiovasc. Pharmacol.* **4,** 935–941
66. Williams, L. T., and Jones, L. R. (1983) *J. Biol. Chem.* **258,** 5344–5347
67. Sutko, J. L., and Kenyon, J. L. (1983) *J. Gen. Physiol.* **82,** 385–404
68. Ohnishi, S. T. (1979) *J. Biochem.* **86,** 1147–1150
69. Miyamoto, H., and Racker, E. (1981) *FEBS Lett.* **133,** 235–238
70. Miyamoto, H., and Racker, E. (1982) *J. Membr. Biol.* **66,** 193–201
71. Yamamoto, N., and Kasai, M. (1982) *J. Biochem.* **92,** 485–496
72. Kirino, Y., and Shimizu, H. (1982) *J. Biochem.* **92,** 1287–1296
73. Kim, D. H., Ohnishi, S. T., and Ikemoto, N. (1983) *J. Biol. Chem.* **258,** 9662–9668
74. Kirino, Y., Osakabe, M., and Shimizu, H. (1983) *J. Biochem.* **94,** 1111–1118
75. Yamamoto, N., and Kasai, M. (1982) *J. Biochem.* **92,** 465–475
76. Weber, A., and Herz, R. (1968) *J. Gen. Physiol.* **52,** 750–759
77. Nagasaki, K., and Kasai, M. (1981) *J. Biochem.* **90,** 749–755
78. Morii, H., and Tonomura, Y. (1983) *J. Biochem.* **93,** 1271–1285
79. Ogawa, Y., and Ebashi, S. (1976) *J. Biochem.* **80,** 1149–1157
80. Takagi, A. (1981) *Exp. Neurol.* **73,** 477–486
81. Shoshan, V., MacLennan, D. H., and Wood, D. S. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 4828–4832
82. Mitchell, R. D., Volpe, P., Palade, P., and Fleischer, S. (1983) *J. Biol. Chem.* **258,** 9867–9877
83. Fairhurst, A. S., and Hasselbach, W. (1970) *Eur. J. Biochem.* **13,** 504–509
84. Bindoli, A., and Fleischer, S. (1983) *Arch. Biochem. Biophys.* **221,** 458–466
85. Chiesi, M., and Carafoli, E. (1982) *J. Biol. Chem.* **257,** 984–991
86. Ehlert, F. J., Itoga, E., Roeske, W. R., and Yamamura, H. I. (1982) *Biochem. Biophys. Res. Commun.* **104,** 937–943
87. De Pover, A., Matlib, M. A., Lee, S. W., Dube, G. P., Grupp, I. L., Grupp, G., and Schwartz, A. (1982) *Biochem. Biophys. Res. Commun.* **108,** 110–117
88. Yamamura, H. I., Schoemaker, H., Boles, R. G., and Roeske, W. R. (1982) *Biochem. Biophys. Res. Commun.* **108,** 640–646
89. Murphy, K. M. M., Gould, R. J., Largent, B. L., and Snyder, S. H. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 860–864
90. Kim, D. H., and Ikemoto, N. (1983) *Biophys.* **41,** 232a
91. Volpe, P., Costello, B., Chu, A, and Fleischer, S. (1984) *Arch. Biochem. Biophys.*, in press