Biochemical Pharmacology, Vol. 34, No. 2, pp. 195–201, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
Pergamon Press Ltd.

STIMULATION OF CANINE CARDIAC SARCOPLASMIC RETICULUM Ca^{2+} UPTAKE BY DIHYDROPYRIDINE Ca^{2+} ANTAGONISTS

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(*Received* 3 *January* 1984; *accepted* 9 *May* 1984)

Abstract—We examined the effects of four Ca^{2+} antagonists that possess the ability to bind to calmodulin—felodipine, nitrendipine, prenylamine, and verapamil—as well as the effect of the calmodulin antagonist trifluoperazine on Ca^{2+} uptake and $Ca^{2+} + Mg^{2+}$/ATPase activity in canine cardiac sarcoplasmic reticulum. In the presence of 20–30 μM felodipine and 100–200 μM nitrendipine, Ca^{2+} uptake increased from 69 nmoles $\cdot mg^{-1} \cdot min^{-1}$ to 107 and 108 nmoles $\cdot mg^{-1} \cdot min^{-1}$, respectively, with half-maximal stimulation occurring at 7.5 and 28 μM respectively. $Ca^{2+} + Mg^{2+}$/ATPase activity was unchanged over the same concentration ranges. In contrast, both Ca^{2+} uptake and $Ca^{2+} + Mg^{2+}$/ATPase activities were inhibited in the presence of 10–100 μM trifluoperazine ($IC_{50} = 25$ μM), 10–100 μM prenylamine ($IC_{50} = 35$ μM) and 100–200 μM verapamil (inhibition insufficient for IC_{50} determination). None of the drugs affected membrane permeability to Ca^{2+} as determined by passive $^{45}Ca^{2+}$ efflux in the presence of ethyleneglycol bis(β-amenoethyl ether)N,N,N^1-tetraacetic acid (EGTA). Drug inhibition of calmodulin-dependent turkey gizzard myosin light chain kinase activation in a purified protein system was used as a direct measure of calmodulin antagonism, and felodipine, nitrendipine, trifluoperazine, prenylamine, and verapamil blocked this activation at IC_{50} values of 9.8, 55, 6.4, 31, and 93 μM respectively. None of the drugs studied, however, had any effect upon endogenous phospholamban phosphorylation in our cardiac sarcoplasmic reticulum preparations. These observations indicate that dihydropyridine Ca^{2+} antagonists stimulate cardiac sarcoplasmic reticulum Ca^{2+} uptake *in vitro* either by increasing the efficiency of the transport process or by inhibiting Ca^{2+}-dependent Ca^{2+} release, and suggest that these effects do not result from interference with calmodulin-mediated processes.

Many of the biochemical processes relating to cell contractility are regulated by changes in the intracellular Ca^{2+} concentration via the protein calmodulin [1]. Binding of Ca^{2+} to calmodulin induces a conformational change in the protein that allows it to bind to and activate enzymes involved in contractile processes such as myosin light chain kinase [2]. A number of drugs whose negative inotropic and chronotropic effects in cardiac muscle have been ascribed to their ability to block Ca^{2+} influx across plasma membranes have been shown to bind to calmodulin in the presence of Ca^{2+} and to inhibit calmodulin-dependent enzyme activation *in vitro* [3–6]. Many of these "Ca^{2+} antagonists" are able to permeate plasma membranes and accumulate within cardiac muscle [7], and several lines of evidence have suggested that these drugs may act intracellularly as well as at the plasma membrane [3, 8–11]. It would seem reasonable to suspect that interference with calmodulin-mediated processes might play a role in these intracellular actions.

ATP-dependent Ca^{2+} uptake by cardiac sarcoplasmic reticulum, a process whereby the intracellular Ca^{2+} concentration and hence the contractile state of cardiac muscle cells is regulated, can be stimulated in association with phosphorylation of the sarcoplasmic reticulum protein phospholamban by a membrane-associated calmodulin-dependent kinase [12–19]. Drugs that bind to calmodulin and inhibit the activation of calmodulin-dependent enzymes might therefore be expected to inhibit sarcoplasmic reticulum Ca^{2+} uptake. Inhibition of cardiac sarcoplasmic reticulum Ca^{2+} uptake by the Ca^{2+} antagonists verapamil and methoxyverapamil has been reported to occur at drug concentrations of 0.1 to 3.0 mM [20–22]. The inhibitory effects of methoxyverapamil appear to be dependent upon the presence of 0.1 M KCl, with the drug having an unexplained stimulatory effect on Ca^{2+} uptake in its absence [22]. In contrast, a stimulation of Ca^{2+} uptake by the dihydropyridine Ca^{2+} antagonists nitrendipine and nimodipine in the presence of 0.12 M KCl has been reported [23]. In none of these cases, however, has the molecular mechanism of action of the drugs or the relation between calmodulin-antagonistic properties and effects upon cardiac sarcoplasmic reticulum been examined.

We studied the effects of four Ca^{2+} antagonists with known calmodulin binding properties—felodipine, nitrendipine, prenylamine, and verapamil—as well as the effects of the calmodulin

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antagonist trifluoperazine on Ca^{2+} uptake, Ca^{2+} efflux, and Ca^{2+} + Mg^{2+}/ATPase* activity in canine cardiac sarcoplasmic reticulum. Inhibition of calmodulin-dependent turkey gizzard myosin light chain kinase activity was studied in a purified protein system as a direct measure of the calmodulin-antagonistic properties of the drugs. Finally, the effects of the drugs on phospholamban phosphorylation were studied directly to determine the correlation between calmodulin antagonism and effects upon cardiac sarcoplasmic reticulum Ca^{2+} uptake.

MATERIALS AND METHODS

Canine cardiac sarcoplasmic reticulum was prepared according to a modification of the method of Harigaya and Schwartz [24] as adapted by Bidlack and Shamoo [25]. Male foxhounds (25–30 kg each) were anesthetized with sodium pentobarbital (30 mg/kg) and killed by phlebotomy; hearts were immediately removed and washed in ice-cold 0.9% NaCl. Left ventricles were trimmed of fat and connective tissue, cut into 1 cm^3 portions, and homogenized in 4–5 vol. (v/w) of 0.29 M sucrose, 1 mM DTT, 3 mM NaN_3, 10 mM Tris–HCl, (pH 7.0, 4°). Sedimentation and washing in 0.6 M KCl were performed as described previously [25], and the final pellet was suspended by hand homogenization in a glass–glass apparatus in 5 ml of the starting sucrose buffer. The protein concentration of the preparation was determined by measuring absorbance at 280 nm in 1% sodium dodecyl sulfate (SDS) [26]. The preparation could be quick frozen in liquid nitrogen and stored at −70° with negligible loss of activity.

Ca^{2+} uptake by canine cardiac sarcoplasmic reticulum was determined according to the method of Van Winkle *et al.* [27] using absorbance by Arsenazo III as a measure of extravesicular Ca^{2+}. Ca^{2+}/calmodulin antagonists were added at various concentrations to a reaction mixture consisting of 5 mM oxalic acid, 5 mM $MgCl_2$, 3 mM NaN_3, 40 μM $CaCl_2$, 0.12 M KCl, 60 μM Arsenazo III sodium salt, 20 μM MOPS (pH 6.8, 25°) and 0.25 mg/ml sarcoplasmic reticulum. In the case of felodipine and nitrendipine, which are poorly soluble in water, drugs were added in the form of stock ethanolic solutions such that the final reaction mixture had an ethanol concentration of 0.5%. Ca^{2+} uptake was initiated by the addition of ATP to a concentration of 2.5 mM, and the difference between absorbance at 674 nm and absorbance at 684 nm was followed continuously (with continuous stirring) for 5 min in a Hewlett–Packard diode array spectrophotometer. The rate of Ca^{2+} uptake in the presence of various concentrations of drug was compared to that of the appropriate aqueous or 0.5% ethanolic control mixture. The presence of 0.5% ethanol had no effect on the rate of Ca^{2+} uptake. Ca^{2+} uptake was completely dependent on the presence of ATP. When oxalate was absent from the reaction mixture, Ca^{2+} uptake was ≤ 9 nmoles $\cdot mg^{-1} \cdot min^{-1}$ in either the presence or absence of drugs.

Ca^{2+} + Mg^{2+}/ATPase activity was measured spectrophotometrically. Sarcoplasmic reticulum vesicles were suspended at a concentration of 0.15 mg/ml in 5 mM oxalic acid, 0.12 M KCl, 3 mM NaN_3, 5 mM $MgCl_2$, 40 μM $CaCl_2$, and 10 mM MOPS (pH 6.8, 25°). ATP hydrolysis was initiated by the addition of ATP to a concentration of 2.5 mM. Ca^{2+}-independent ATPase activity was measured by including 0.5 mM EGTA in place of 40 μM $CaCl_2$ in the reaction mixture. Aliquots were removed at 30-sec intervals for 2 min, and the reaction was quenched by addition of trichloroacetic acid to a final concentration of 10%. The aliquots were centrifuged, and inorganic phosphate in the supernatant fraction was estimated by the method of LeBel *et al.* [28]. Assays with felodipine and nitrendipine were done in the presence of 0.5% ethanol, which had no significant effect upon Ca^{2+} + Mg^{2+}/ATPase activity.

Ca^{2+} efflux was determined using filtration methods involving $^{45}Ca^{2+}$ loading. ATP to a concentration of 2.5 mM was added to a solution containing 5 mM oxalic acid, 0.12 M KCl, 3 mM NaN_3, 5 mM $MgCl_2$, 40 μM $^{45}CaCl_2$ (3 mCi/mmole), 0.25 mg/ml sarcoplasmic reticulum, and 20 mM MOPS, (pH 6.8, 25°; final volume 1.0 ml). At 5 min, Ca^{2+} uptake was stopped by the addition of EGTA to a concentration of 1.0 mM. Ca^{2+}/calmodulin antagonist or the divalent-cation specific ionophore A23187 was then added to the reaction mixture, and 180 μl aliquots were removed at 1-min intervals thereafter and filtered on a Millipore apparatus under suction through Whatman cellulose nitrate filter discs (pore size 0.2 μm). In the case of the dihydropyridines and A23187, the final solution contained 0.5% ethanol, and measurements were standardized against solutions containing 0.5% ethanol with no added drug. Each filter disc was washed three times with 2 ml buffer containing 5 mM oxalic acid, 0.12 M KCl, 3 mM NaN_3, 5 mM $MgCl_2$, and 20 mM MOPS, (pH 6.8, 25°), and radioactivity adhering to each filter disc was measured in 10 ml ACS (Amersham) in a Beckman LS 7500 scintillation spectrometer.

Myosin light chain kinase activity was assayed according to the method of Adelstein and Klee [29] as modified by Movsesian *et al.* for determination of drug inhibition [6]. Calmodulin and myosin light chain kinase were present at concentrations of 10^{-9} M and 6.4×10^{-9} M to 10×10^{-9} M, respectively, ensuring that the reaction rate was limited by the calmodulin concentration. Phosphate incorporation into myosin light chains was followed at 1-min intervals over a 5-min time course (during which time myosin light chain phosphorylation proceeded linearly) in the presence and absence of various concentrations of Ca^{2+}/calmodulin inhibitors.

Phosphorylation of phospholamban was performed in a reaction mixture containing cardiac sarcoplasmic reticulum suspended at a concentration of 1.0 mg/ml in 5 mM $MgCl_2$, 6 mM NaN_3, 5 mM NaF, 0.2 mM $CaCl_2$, and 40 mM MOPS (pH = 6.8,

* Abbreviations: Ca^{2+} + Mg^{2+}/ATPase, Ca^{2+}- and Mg^{2+}-dependent ATP hydrolyzing activity; DTT, dithiothreitol; MOPS, [*N*-morpholino]-propanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetra-acetic acid; and Tris–HCl, Tris(hydroxymethyl)aminomethane hydrochloride.

27°). Calmodulin and Ca^{2+}/calmodulin antagonists were present as indicated in the figure legends; the presence of ethanol at 0.5% (v/v) had no effect on phosphorylation under these conditions. Phosphorylation was initiated by the addition of [γ-^{32}P]ATP to a concentration of 0.05 mM. The reaction was stopped at 4.5 min by the addition of an equal volume of SDS buffer, and aliquots were electrophoresed in 1% SDS–7.5–20% polyacrylamide slab gels according to the method of Laemmli [30]. Prior to electrophoresis, one-half of each reaction mixture was heated at 95° for 5 min. Phospholamban was identified as a phosphoprotein band migrating with an apparent molecular weight of 27,000 following solubilization at room temperature and 5,000 following solubilization at 95°. Phosphoprotein bands were identified by autoradiography using Kodak X-omat AR film; ^{32}P-incorporation was quantified by excising the bands and measuring radioactivity as described above.

Felodipine was a gift from AB Hassle (Sweden). Nitrendipine was a gift from Miles Laboratories. Verapamil hydrochloride was a gift from Knoll Pharmaceuticals. Prenylamine lactate was a gift from Hoechst-Roussel Pharmaceuticals. Trifluoperazine hydrochloride was a gift from Smith Kline & French Laboratories. Arsenazo III sodium salt was purchased from Sigma. $^{45}CaCl_2$ was purchased from New England Nuclear. Foxhounds were obtained from a breeding farm operated by the Veterinary Research Branch of the Division of Research Services, National Institutes of Health.

RESULTS

The effects of Ca^{2+}/calmodulin antagonists on Ca^{2+} uptake and Ca^{2+} + Mg^{2+}/ATPase activities of canine cardiac sarcoplasmic reticulum are shown in Figs. 1 and 2. The dihydropyridines felodipine and nitrendipine stimulated cardiac sarcoplasmic reticulum Ca^{2+} uptake. Stimulation by felodipine reached a maximum of 55% in the concentration range of 20–30 μM ($EC_{50} \sim 7.5$ μM) while stimulation by nitrendipine appeared to reach a plateau of 57% in the concentration range of 100–200 μM ($EC_{50} \sim 28$ μM). In the case of felodipine, inhibition of Ca^{2+} uptake was seen at drug concentrations ≥ 100 μM, an effect which could not be studied with nitrendipine due to its insolubility at higher concentrations. Dihydropyridine stimulation of Ca^{2+} uptake activity was unaccompanied by any increase in Ca^{2+} + Mg^{2+}/ATPase activity, and the effects of the dihydropyridines on the ratio of Ca^{2+} uptake to ATP hydrolysis are given in Table 1. In contrast, Ca^{2+} uptake was inhibited by trifluoperazine, prenylamine, and, to a lesser extent, verapamil over the concentration ranges of 10–100 μM ($IC_{50} \sim 25$ μM), 10–100 μM ($IC_{50} \sim 35$ μM), and 100–200 μM (inhibition insufficient for IC_{50} determination) respectively. For each of these drugs, inhibition of Ca^{2+}

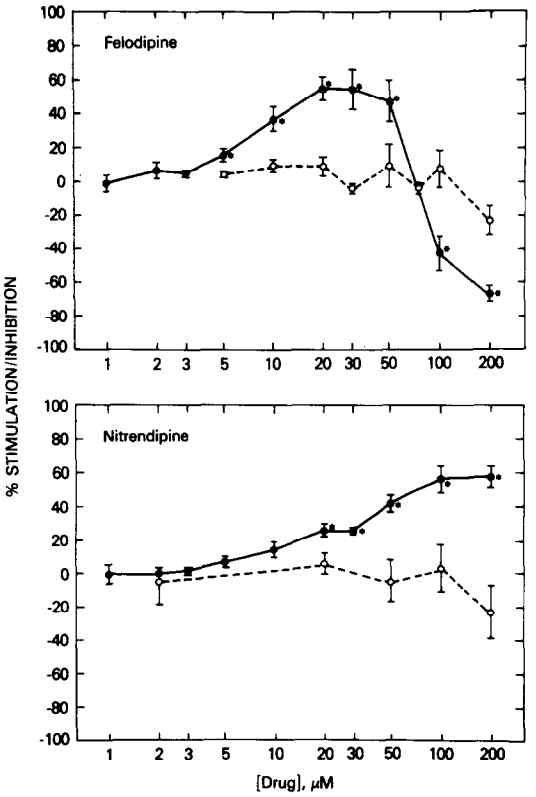

Fig. 1. Stimulation of canine cardiac sarcoplasmic reticulum Ca^{2+} uptake by dihydropyridine Ca^{2+}/calmodulin antagonists. Ca^{2+} uptake (●———●) and Ca^{2+} + Mg^{2+}/ATPase (○— — —○) activities were assayed as described in Methods in the presence of various drug concentrations. Drug effects are expressed as percent stimulation or inhibition of control values (see Table 1). Each point represents the mean ± S.E. of four to eight determinations from at least two membrane preparations. Points different from control with a P values ≤ 0.05 are indicated with an asterisk.

Table 1. Effects of dihydropyridines on Ca^{2+} uptake and Ca^{2+} + Mg^{2+}/ATPase activities of canine cardiac sarcoplasmic reticulum, showing the coupling of Ca^{2+} uptake to ATP hydrolysis*

	Ca^{2+} uptake†	Ca^{2+} + Mg^{2+}/ ATPase‡	Moles Ca^{2+}/ mole ATP
Control	69 ± 3	122 ± 13	0.57 (0.48–0.66)
Felodipine, 30 μM	107 ± 8	118 ± 4	0.90 (0.87–1.00)
Nitrendipine, 100 μM	108 ± 5	127 ± 17	0.85 (0.72–1.03)

* Values are mean ± S.E. for four to eight determinations. Controls were done in the presence of 0.5% ethanol (v/v).

† Expressed in nmoles $Ca^{2+} \cdot mg^{-1} \cdot min^{-1}$.

‡ Expressed in nmoles $PO_4^{-4} \cdot mg^{-1} \cdot min^{-1}$.

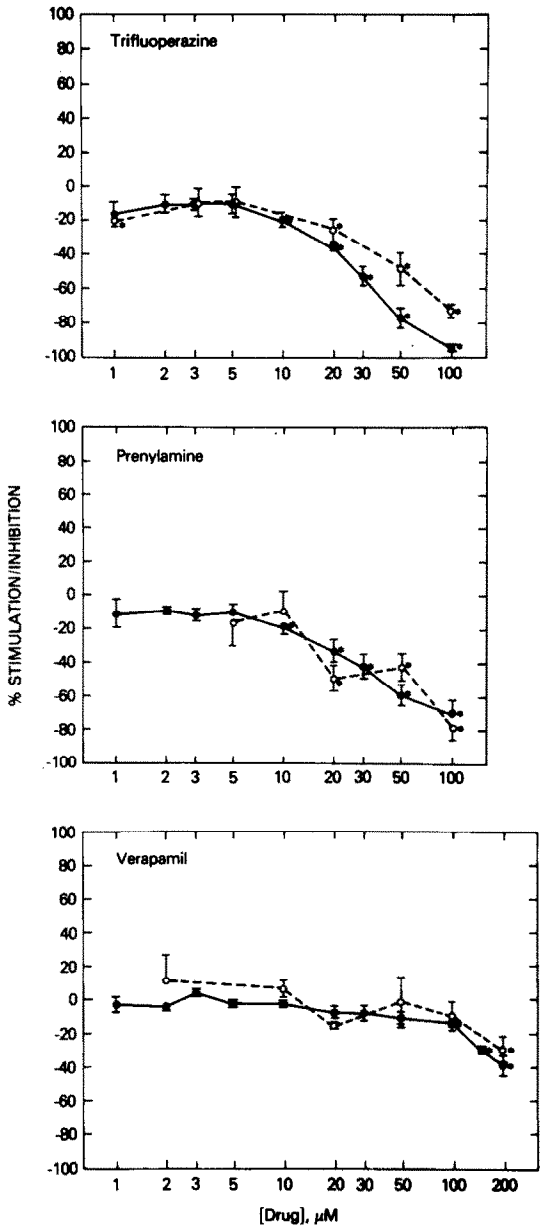

Fig. 2. Inhibition of canine cardiac sarcoplasmic reticulum Ca^{2+} uptake by non-dihydropyridine Ca^{2+}/calmodulin antagonists. Effects on Ca^{2+} uptake (●——●) and Ca^{2+} + Mg^{2+}/ATPase (○— — —○) activities are shown as explained for Fig. 1.

uptake was accompanied by comparable inhibition of Ca^{2+} + Mg^{2+}/ATPase activity in the same concentration range.

To determine whether effects on Ca^{2+} uptake were the result of drug-induced changes in the permeability of cardiac sarcoplasmic reticulum to Ca^{2+}, we examined the effects of the Ca^{2+}/calmodulin antagonists on Ca^{2+} efflux (Fig. 3). Membrane vesicles loaded with $^{45}Ca^{2+}$ were treated with EGTA, which completely inhibited Ca^{2+} uptake, and $^{45}Ca^{2+}$ efflux was followed over a 5-min period after addition of drug. No efflux was observed under

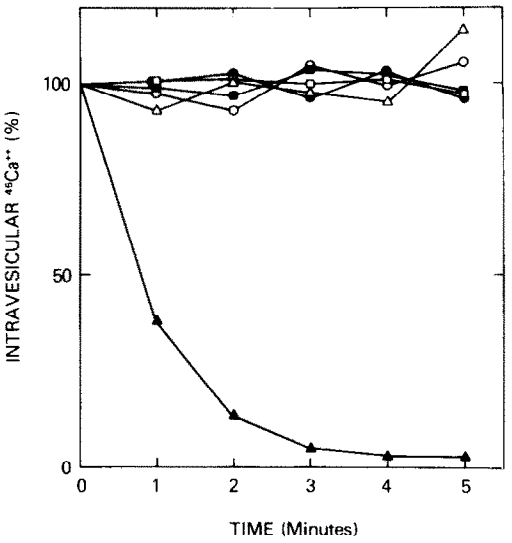

Fig. 3. Effect of Ca^{2+}/calmodulin antagonists on passive $^{45}Ca^{2+}$ efflux from canine cardiac sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles were loaded with $^{45}Ca^{2+}$ and treated with 100 μM trifluoperazine (○), 30 μM felodipine (■), 100 μM prenylamine (△), 100 μM nitrendipine (●), 200 μM verapamil (□), or 10 μM A23187 (▲) as described in Methods $^{45}Ca^{2+}$ remaining within the vesicles, expressed as a percentage of the vesicular $^{45}Ca^{2+}$ content prior to addition of drug, was followed over time. Each point represents the mean of two determinations. All points for A23187 differ from control with a P value ≤0.05.

these conditions in either the absence or presence of 100 μM trifluoperazine, 200 μM verapamil, 100 μM prenylamine, 30 μM felodipine, or 100 μM nitrendipine. In contrast, when the divalent cation-specific ionophore A23187 was added to the reaction mixture at a concentration of 10 μM, Ca^{2+} efflux proceeded briskly.

As part of our effort to determine whether drug effects on sarcoplasmic reticulum Ca^{2+} uptake correlated with drug inhibition of calmodulin-dependent enzyme activation, we examined the inhibition of calmodulin-dependent turkey gizzard myosin light chain kinase activity by Ca^{2+}/calmodulin antagonists in a purified protein system (Fig. 4). Previous studies have demonstrated that, under conditions in which the rate of myosin light chain phosphorylation is limited by the calmodulin concentration, the concentration of calmodulin antagonist giving 50% inhibition of myosin light chain kinase activity is similar to the apparent dissociation constant for that drug with calmodulin [6]. Under the conditions described in Methods, trifluoperazine, felodipine, prenylamine, nitrendipine, and verapamil gave 50% inhibition of calmodulin-dependent myosin light chain kinase activity at concentrations of 6.4, 9.8, 31, 55, and 93 μM respectively.

Finally, in order to determine the relation between calmodulin antagonism and effects upon Ca^{2+} uptake, we studied the effects of the Ca^{2+}/calmodulin antagonists on the phosphorylation of phospholamban in cardiac sarcoplasmic reticulum (Fig. 5). Phosphorylation of phospholamban was minimal in

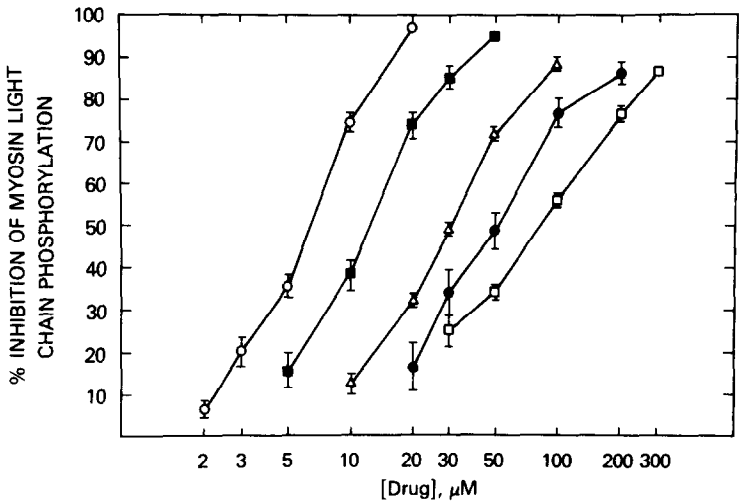

Fig. 4. Inhibition of calmodulin-dependent turkey gizzard myosin light chain kinase activity by Ca^{2+}/calmodulin antagonists. Myosin light chain kinase activity was assayed as described in Methods and inhibition by various concentrations of trifluoperazine (○), felodipine (■), prenylamine (△), nitrendipine (●), and verapamil (□) is expressed relative to the level of myosin light chain kinase activity in the absence of drug. Each point represents the mean ± S.E. of three to eight determinations. The data for felodipine and nitrendipine are taken from Movsesian *et al.* [6]. All points except 2 μM trifluoperazine differ from control with a P value ≤0.05.

our preparations in the absence of exogenous calmodulin and was unaffected by the presence of 30 μM felodipine, 100 μM nitrendipine, 100 μM trifluoperazine, 200 μM verapamil, or 100 μM prenylamine. In contrast, addition of 0.5 μM exogenous calmodulin led to a 12-fold stimulation of phospholamban phosphorylation.

DISCUSSION

Stimulation of cardiac sarcoplasmic reticulum Ca^{2+} uptake by calmodulin is believed to occur through the calmodulin-dependent phosphorylation of phospolamban by a membrane-bound protein kinase [12–19]. Our expectation in designing these

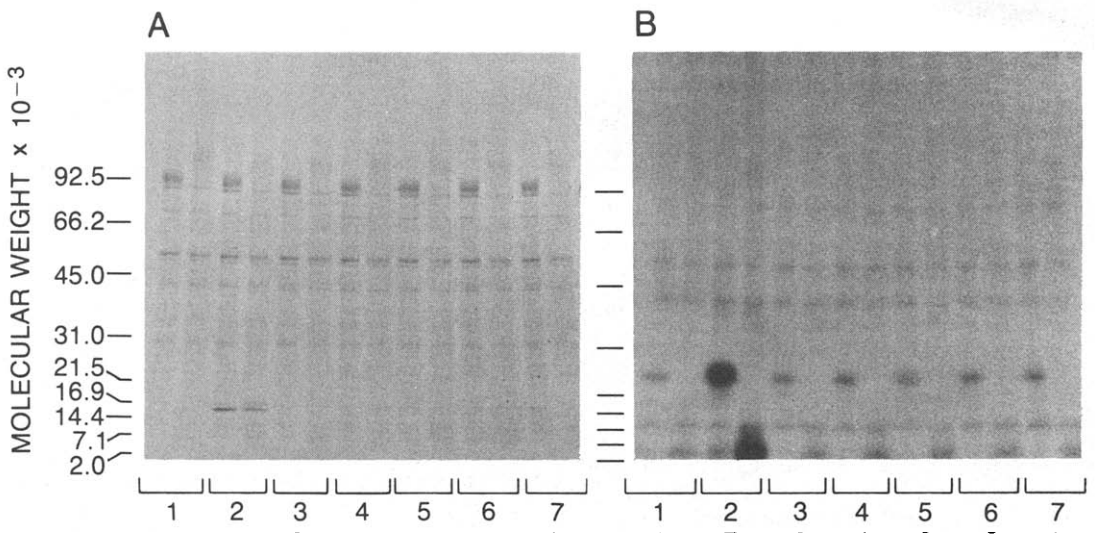

Fig. 5. Effects on Ca^{2+}/calmodulin antagonists on phospholamban phosphorylation in cardiac sarcoplasmic reticulum. Membrane vesicles were phosphorylated and applied to SDS-polyacrylamide slab gels as described in Methods. The left lane of each numbered pair contained samples applied following solubilization at room temperature; the right lane contained samples applied following solubilization at 95° × 5 min. Coomassie blue stain (A) and autoradiograph (B) are shown. The 27,000 M_r band in each lane pair was excised and counted. Key: (1) control (173 cpm); (2) 0.5 μM calmodulin (2121 cpm); (3) 30 μM felodipine (173 cpm); (4) 100 μM nitrendipine (212 cpm); (5) 100 μM trifluoperazine (131 cpm); (6) 100 μM prenylamine (198 cpm); and (7) 200 μM verapamil (212 cpm).

experiments was that drugs that bind to calmodulin and block the activation of calmodulin-dependent enzymes might have effects upon Ca^{2+} uptake by cardiac sarcoplasmic reticulum attributable to inhibition of calmodulin-dependent phosphorylation of phospholamban. Trifluoperazine, prenylamine, and verapamil were found to inhibit Ca^{2+} uptake, and this inhibition was associated with inhibition of $Ca^{2+} + Mg^{2+}$/ATPase activity. In contrast, the dihydropyridines felodipine and nitrendipine were found to stimulate Ca^{2+} uptake over a range of drug concentrations, and this stimulation occurred without stimulation of $Ca^{2+} + Mg^{2+}$/ATPase activity. Our findings differ from those of Colvin *et al.*, who previously reported that stimulation of Ca^{2+} uptake in cardiac sarcoplasmic reticulum by 10 μM nitrendipine is accompanied by an increased in $Ca^{2+} + Mg^{2+}$/ATPase activity [23]; the reason for the difference between their observation and ours is unclear.

The stimulation of cardiac sarcoplasmic reticulum Ca^{2+} uptake by dihydropyridine Ca^{2+} antagonists in the absence of a stimulation of $Ca^{2+} + Mg^{2+}$/ATPase activity is reminiscent of reports of similar observations with the agents ryanodine and ruthenium red [31–34], and can be explained by either of two possibilities. First, the drugs might interact directly with one of the components of the Ca^{2+} transport system and cause an increase in the efficiency of the Ca^{2+} uptake process. Alternatively, as Ca^{2+} uptake represents a balance between Ca^{2+} influx and Ca^{2+} efflux, these effects might result from inhibition by the drugs of the latter process. Our studies indicated that no passive Ca^{2+} efflux occurred under experimental conditions similar to those used to study Ca^{2+} uptake in either the absence or presence of the drugs, implying that drug effects cannot be ascribed to changes in membrane permeability to Ca^{2+}. Intravesicular Ca^{2+} can be released from cardiac sarcoplasmic reticulum by extravesicular Ca^{2+} [35–39], however, and this Ca^{2+}-mediated release might occur concurrently with active Ca^{2+} transport and thereby affect the rate of Ca^{2+} uptake. As our Ca^{2+} efflux studies were done in the presence of excess extravesicular EGTA, the extent of Ca^{2+}-mediated Ca^{2+} efflux occurring during Ca^{2+} uptake determinations and the possible inhibition of this process by dihydropyridine Ca^{2+} antagonists in our preparation cannot be assessed. Thus, as with ryanodine or ruthenium red, whether the effects of dihydropyridine Ca^{2+} antagonists result principally from a direct stimulation of Ca^{2+} influx or from inhibition of Ca^{2+}-mediated Ca^{2+} efflux cannot be determined.

It would seem reasonable to suspect that the effects of Ca^{2+}/calmodulin antagonists on cardiac sarcoplasmic reticulum Ca^{2+} uptake might be attributable to the ability of the drugs to bind to calmodulin and interfere with calmodulin-mediated processes. This possibility seemed especially plausible in light of the similar drug concentration ranges over which effects on cardiac sarcoplasmic reticulum Ca^{2+} uptake and inhibition of calmodulin-dependent turkey gizzard myosin light chain kinase activity were observed. Our direct study of phospholamban phosphorylation in cardiac sarcoplasmic reticulum revealed, however, that phospholamban phosphorylation in our preparations was largely dependent upon the addition of exogenous calmodulin and that the drugs tested had no significant effect upon the phosphorylation of phospholamban in its absence. The effects of the Ca^{2+}/calmodulin antagonists on cardiac sarcoplasmic reticulum function reported here thus do not appear to be mediated by interference with calmodulin-dependent phospholamban phosphorylation, and the similar drug concentration ranges over which effects on Ca^{2+} uptake and inhibition of calmodulin-dependent enzyme activation occur is probably coincidental. Interestingly, trifluoperazine inhibits Ca^{2+} uptake and Ca^{2+}/ATPase activities in skeletal muscle sarcoplasmic reticulum over a concentration range virtually identical to that affecting these activities in cardiac sarcoplasmic reticulum, and the effects in skeletal muscle also appear to be calmodulin-independent [40–42]. Whether the drug acts through a similar mechanism in both systems remains to be determined.

It is unclear whether the *in vitro* effects of Ca^{2+} antagonists on cardiac sarcoplasmic reticulum Ca^{2+} uptake reported here contribute to the *in vivo* negative inotropic properties of these agents. The effects of felodipine and nitrendipine on cardiac sarcoplasmic reticulum described here occur at concentrations several orders of magnitude higher than those required for binding of dihydropyridines to plasma membranes and inhibition of Ca^{2+} influx, although a more recent publication documented half-maximal inhibition of plasma membrane Ca^{2+} influx occurring at a nitrendipine concentration of 0.154 μM [43–45]. Dihydropyridines have also been found to bind cardiac sarcoplasmic reticulum with equilibrium dissociation constants of 0.2 to 0.4 nM, although this binding did not correlate with changes in transmembrane Ca^{2+} flux [46]. On the other hand, dihydropyridine Ca^{2+} antagonists are quite hydrophobic, and the concentrations achieved within cardiac sarcoplasmic reticulum *in vivo* following long-term administration have not been determined. Whether these drugs may additionally have any effects upon Ca^{2+} uptake by smooth muscle sarcoplasmic reticulum or upon extracellular Ca^{2+} extrusion by the Ca^{2+}/ATPase of the myocardial plasma membrane has likewise not been determined. Regardless of their pertinence to the therapeutic mechanisms of action of these drugs, however, our observations suggest that Ca^{2+} antagonists, in general, and dihydropyridines, in particular, may prove to be valuable as probes for studying the molecular mechanisms involved in the regulation of Ca^{2+} uptake by cardiac sarcoplasmic reticulum.

Acknowledgements—The authors are indebted to Rodney Green and George Alfieris for their expert technical assistance, to Rodney Levine for sharing his expertise in spectrophotometric methods, to Michael Beaven for his editorial advice, and to Imogene Surrey for her assistance in preparing the manuscript. This work was supported in part by the Department of Energy (DEAS05050580 EV10329).

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