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INHIBITION OF TURKEY GIZZARD MYOSIN LIGHT CHAIN KINASE ACTIVITY BY DIHYDROPYRIDINE CALCIUM ANTAGONISTS*

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Abstract—Drugs which block the influx of calcium (Ca^{2+}) across plasma membranes may additionally have direct effects upon smooth muscle contractile proteins. In a system of purified proteins comprised of calmodulin, turkey gizzard myosin light chains, and turkey gizzard myosin light chain kinase, the inhibition of myosin light chain phosphorylation by the dihydropyridine Ca^{2+} antagonists felodipine [3-ethyl-5-methyl-1-1,4-dihydro-2,6-dimethyl-4-(2,3-dichlorophenyl)-3,5-pyridine dicarboxylate] and nitrendipine [3-ethyl-5-methyl-1-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate] was studied. In the presence of excess myosin light chain kinase, 50% inhibition of myosin light chain phosphorylation occurred at felodipine and nitrendipine concentrationsof $9.8 \pm 1.1 \times 10^{-6}$ M and $5.6 \pm 0.6 \times 10^{-5}$ M respectively. Inhibition of light chain kinase activity could not be overcome by increasing the free Ca^{2+} concentration from 0.05 to 5.0 mM. Felodipine was unable to inhibit the activity of myosin light chain kinase rendered Ca^{2+}/calmodulin-independent by limited tryptic digestion. Using molecular sieve chromatography, nitrendipine was found to bind to calmodulin with an apparent dissociation constant (K_{app}) of $5.2 \pm 0.3 \times 10^{-5}$ M, and this binding was Ca^{2+} dependent. These data suggest that dihydropyridines inhibit the phosphorylation of smooth muscle myosin light chains *in vitro* by binding to Ca^{2+}/calmodulin and inhibiting the activation of myosin light chain kinase.

"Calcium antagonist" drugs block the influx of Ca^{2+} across cell membranes, and their ability to inhibit cardiac and smooth muscle contractility has generally been ascribed to this property [1]. In several smooth muscle preparations, however, Ca^{2+} antagonists have been shown to cause relaxation at doses lower than those required to inhibit Ca^{2+} influx and/or action potentials [2, 3] and to inhibit contractions induced in the absence of extracellular Ca^{2+} [4]. Furthermore, the Ca^{2+} antagonist diltiazem has been shown to reduce the amplitude of contraction obtainable at any given level of Ca^{2+}-dependent membrane depolarization [5]. Finally, it has been demonstrated that Ca^{2+} antagonists can accumulate in smooth muscle [6], and that while the membrane-permeable Ca^{2+} antagonist methoxyverapamil is effective when applied either intracellulary or extracellularly, its membrane-impermeable quaternary amine derivative is effective only following direct intracellular injection [7]. These observations suggest that the smooth muscle relaxant effects of Ca^{2+} antagonists may derive in part from intracellular mechanisms of action and possibly involve direct interactions with the contractile proteins.

Ca^{2+} regulation of smooth muscle contraction is mediated in large measure through the Ca^{2+} binding protein calmodulin (molecular weight = 16,500 daltons) (for review, see Ref. 8). As the intracellular Ca^{2+} concentration rises from 10^{-7} M to 10^{-5} M, binding of Ca^{2+} to calmodulin increases, causing the Ca^{2+}/calmodulin complex to bind to and activate myosin light chain kinase (MLCK‡). This enzyme, inactive unless bound to calmodulin, catalyzes the phosphorylation of the 20,000 dalton light chain to smooth muscle myosin necessary for the actin activation of its Mg^{2+}-ATPase [9, 10]. A number of drugs are able to bind to calmodulin and thereby inhibit the function of calmodulin-dependent enzymes *in vitro* [11], raising the possibility that Ca^{2+} antagonists may act through a similar mechanism. The dihydropyridine Ca^{2+} antagonist felodipine has been shown to bind to proteins of molecular weight 15,000–20,000 daltons in crude extracts from smooth muscle [3]. Changes in nuclear magnetic reasonance spectra and in fluorescence spectra of hydrophobic probes associated with calmodulin in the presence of these drugs, as well as inhibition of calmodulin-dependent enzymes by Ca^{2+} antagonists, have also been demonstrated [3, 12–14]. We therefore examined the effects of felodipine and its analogue nitrendipine (Fig. 1) on MLCK-catalyzed myosin light chain phosphorylation in a system of purified smooth muscle contractile proteins. To determine the mechanism of inhibition, a Ca^{2+}/calmodulin-independent form of MLCK was prepared by limited proteolysis.

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‡ Abbreviations: MLCK, myosin light chain kinase; Mg^{2+}-ATPase, Mg^{2+}-dependent ATP hydrolyzing activity; DTT, dithiothreitol; MOPS, [*N*-morpholino]propanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; and DMSO, dimethyl sulfoxide.

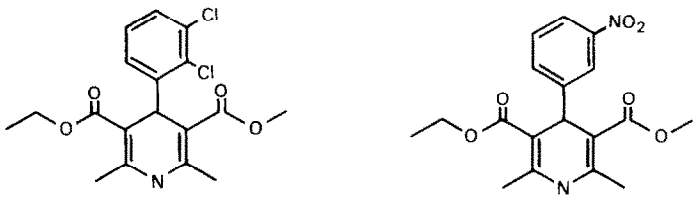

Fig. 1. Structures of felodipine and nitrendipine.

In addition, we examined dihydropyridine–calmodulin binding directly in order to determine its correlation with inhibition of calmodulin-dependent MLCK activation. While the more light stable felodipine was used for most of the experiments, nitrendipine was used for binding studies because of the difficulty in obtaining felodipine in radioactively labeled form.

MATERIALS AND METHODS

Protein preparation. Smooth muscle MLCK was prepared from turkey gizzard by the method of Adelstein and Klee [15]. The enzyme had a V_{max} of 7–15 μmoles/mg-min. Turkey gizzard myosin light chains were purified by the method of Perrie and Perry [16] as described by Adelstein and Klee [15]. As this preparation tended to yield an approximately 1:1 mixture of 17,000 dalton (non-phosphorylatable) and 20,000 dalton (phosphorylatable) light chains, the concentration of the latter was determined by measuring MLCK-catalyzed phosphate incorporation, assuming a stoichiometry of one mole phosphate per mole 20,000 dalton myosin light chain. Calmodulin was purified from bovine brain by the method of Klee [17] and from bovine testis by the method of Yazawa [18]. Ca^{2+}/calmodulin-independent MLCK was prepared by limited proteolysis following a modification of methods described by Hartshorne *et al.* [19] and Adelstein *et al.* [20]. MLCK and calmodulin, at concentrations of 1.7×10^{-7} M and 2.0×10^{-7} M, respectively, were treated with trypsin (1 mg trypsin:44 mg MLCK) on ice in a mixture containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.5), 0.5 mM $CaCl_2$ and 1 mM DTT. The reaction was terminated at 20 min by addition of soybean trypsin inhibitor in 5-fold excess by mass relative to trypsin. No further purification of the reaction product was performed.

Enzyme assays. Phosphorylation of 20,000 dalton myosin light chain by MLCK was assayed as described by Adelstein and Klee [15] at 25° in a 0.2-ml mixture containing 50 mM MOPS (pH 7.3), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. [γ-^{32}P]ATP and 20,000 dalton myosin light chains were present at concentrations of 10^{-4} M and 5×10^{-6} M, respectively, while $CaCl_2$ was present in concentrations ranging from 0.1 to 5.0 mM. The concentrations of calmodulin and MLCK used in the assay mixtures were 10^{-9} M and 6.4×10^{-9} M, respectively, in order to ensure that the reaction rate was limited by the calmodulin concentration (raising the calmodulin concentration above 10^{-9} M was found to increase the reaction rate proportionately, while raising the MLCK concentration above 6.4×10^{-9} M had no effect). When assaying the phosphorylation by Ca^{2+}/calmodulin-independent MLCK, the procedure was altered slightly, in that an aliquot of the proteolyzed reaction mixture containing calmodulin and trypsin-digested MLCK was added to the assay mixture to yield final calmodulin and MLCK concentrations of 8.0×10^{-8} M and 6.8×10^{-8} M respectively. All other conditions were as described above save for the addition of EGTA to a concentration of 2.0 mM to some of the reaction mixtures. Felodipine and nitrendipine, whose solubilities in water are minimal, were dissolved in DMSO and added to the assay mixture to a final DMSO concentration of 5%; this concentration of DMSO was found to have no effect upon MLCK activity. Drug solutions were prepared fresh at the start of each assay in sodium borosilicate tubes and were added to the assay mixtures (also in borosilicate tubes) using glass micropipettes and a micro/pettor (Scientific Manufacturing Industries, Inc.). Care was taken to ensure that solutions containing dihydropyridines did not come into contact with plastic (to which they adsorb) during the course of the assay.

MLCK activity was assayed at 1-min intervals over a 5-min time course, throughout which period phosphate incorporation into myosin light chains proceeded linearly. The reaction was terminated by application of an aliquot of the reaction mixture to a Whatman grade 3MM 2.3 cm filter disc, which was then immersed immediately in ice-cold 10% trichloroacetic acid with 8% sodium pyrophosphate and washed three times in 10% trichloroacetic acid with 2% sodium pyrophosphate. Radioactivity adhering to filter discs was measured in a Beckman LS 7500 scintillation counter following immersion of discs in 10 ml Aquasol (New England Nuclear, Boston, MA, U.S.A.).

The inhibition by a given concentration of drug of MLCK activity was determined by dividing the difference between the reaction rates in the absence and presence of drug by the rate in the absence of drug. Determination of the drug concentration producing 50% inhibition (IC_{50}) was based upon a computer fit of the data according to the Marquardt compromise [21].

Calmodulin binding to nitrendpine was determined according to the method of Hummel and Dreyer [22] as adapted by Hidaka *et al.* [23]. Cal-

modulin was applied to a Sephadex G-25 column (1.0 × 13.2 cm) equilibrated at room temperature with 50 mM MOPS (pH 7.3), 50 mM NaCl, 5% DMSO, 0.1 mM $CaCl_2$ or 1.0 mM EGTA, and concentrations of [5-methyl-^{3}H]nitrendipine ranging from 10.0 to 30.0 μM. Eluent fractions of 0.2 ml were collected by gravity at a flow rate of 0.2 ml/min and were assayed for protein content by the method of Lowry *et al.* [24] as well as for radioactivity by scintillation counting as described above. Binding of [5-methyl-^{3}H]nitrendipine to calmodulin was determined by measuring the increase in radioactivity in protein-containing fractions. The specific activity of the labeled nitrendipine was 100 mCi/mmole.

Felodipine was a gift from AB Hassle (Molndal, Sweden). Nitrendipine was a gift from Miles Pharmaceuticals (West Haven, CT). [5-methyl-^{3}H] Nitrendipine and [γ-^{32}P]ATP were purchased from New England Nuclear. Trypsin and soybean trypsin inhibitor were purchased from Sigma (St. Louis, MO, U.S.A.).

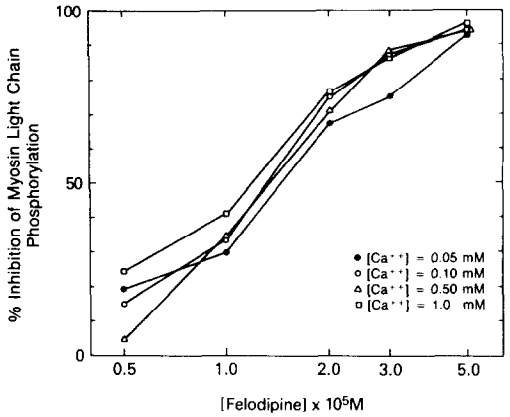

Fig. 3. Effect of [Ca^{2+}] on felodipine-induced inhibition of myosin light chain phosphorylation. Data representing a 20-fold range in [Ca^{2+}] is shown, over which range no significant change in the concentration–inhibition curve was observed.

RESULTS

The inhibition of MLCK-catalyzed myosin light chain phosphorylation by felodipine and nitrendipine was studied as shown in Fig. 2. When the rate of myosin light chain phosphorylation was limited by the calmodulin concentration, felodipine inhibited with an IC_{50} of $9.8 \pm 1.1 \times 10^{-6}$ M. Inhibition was absent at 10^{-6} M felodipine and was essentially complete at 10^{-4} M felodipine. Nitrendipine inhibited myosin light chain phosphorylation less potently, with an IC_{50} of $5.6 \pm 0.6 \times 10^{-5}$ M.

To determine whether dihydropyridine inhibition of myosin light chain phosphorylation is competitive with Ca^{2+}, we examined the effect of increasing the Ca^{2+} concentration on felodipine-induced inhibition (Fig. 3). Increasing the free Ca^{2+} concentration from 0.05 to 1.0 mM did not alter the concentration–inhibition curve significantly. Increasing the Ca^{2+} concentration further to 5.0 mM caused a decrease in MLCK activity, but had no effect on the relative inhibition by various concentrations of felodipine. Thus, felodipine at inhibitory concentrations does not compete with the binding of Ca^{2+} to calmodulin.

To test the possibility that dihydropyridine inhibition of myosin light chain phosphorylation involves inhibition of the activation of MLCK by calmodulin, a Ca^{2+}/calmodulin-independent form of MLCK was prepared by limited tryptic digestion of the enzyme in the presence of bound calmodulin. This treatment, which removes the calmodulin binding site from MLCK, yields a 70,000 dalton peptide which retains full enzymic activity but does not require Ca^{2+}/calmodulin binding for activation. While felodipine inhibited the activity of Ca^{2+}/calmodulin-dependent MLCK, it failed to inhibit the activity of the Ca^{2+}/calmodulin-independent MLCK tryptic digestion product at drug concentrations as high as 5×10^{-4} M (Fig. 4). When Ca^{2+}/calmodulin-dependent and Ca^{2+}/calmodulin-independent MLCK activity

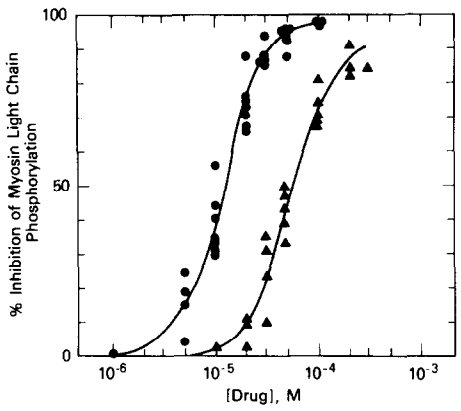

Fig. 2. Inhibition of smooth muscle myosin light chain phosphorylation by felodipine (●) and nitrendipine (▲). Activity of smooth muscle MLCK was assayed by measuring radioactive phosphate (^{32}P) incorporation into smooth muscle myosin light chains in a reaction mixture consisting of 50 mM MOPS (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM $CaCl_2$ and 5% DMSO. Calmodulin and MLCK were present at concentrations of 10^{-9} M and 6.4×10^{-9} M respectively; phosphorylatable myosin light chains and [γ-^{32}P]ATP were present at 5×10^{-6} M and 10^{-4} M respectively.

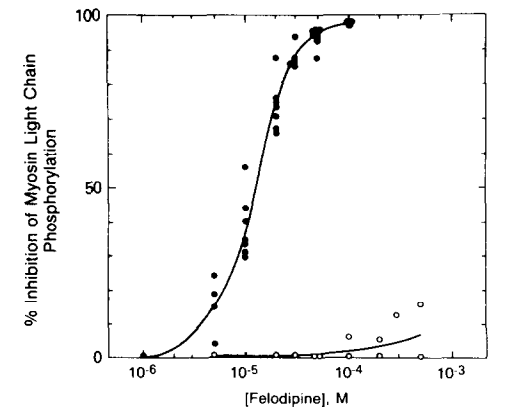

Fig. 4. Felodipine inhibition of Ca^{2+}/calmodulin-dependent (●) and Ca^{2+}/calmodulin-independent (○) MLCK activity. Assay conditions were as described previously.

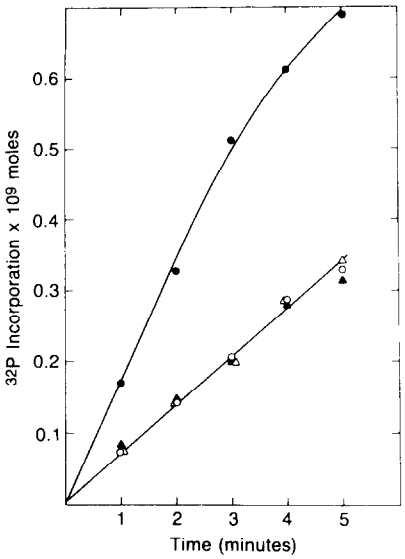

Fig. 5. Effect of partial tryptic digestion of MLCK on felodipine sensitivity. Following limited tryptic digestion, MLCK activity was assayed in the presence of 0.5 mM Ca^{2+} (●), 1.5 mM EGTA (▲), 0.5 mM Ca^{2+} and 0.5 mM felodipine (○), and 1.5 mM EGTA and 0.5 mM felodipine (△). In this experiment, approximately 45% of the MLCK activity was rendered Ca^{2+}/calmodulin-independent. The specific activity of the enzyme in the presence of Ca^{2+} was 6.0 μmoles/mg-min.

were present simultaneously in the assay mixture, felodipine inhibited Ca^{2+}-dependent activity without inhibiting Ca^{2+}-independent activity (assayed in the presence of a 1.5 mM excess of EGTA relative to Ca^{2+}) (Fig. 5). Thus, removal of the calmodulin-binding site from MLCK results in a corresponding loss of felodipine sensitivity.

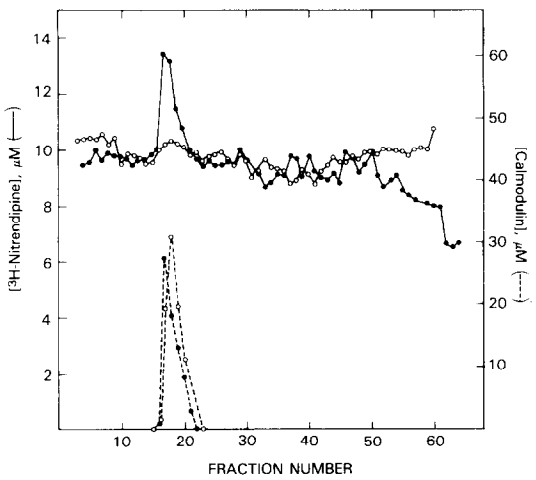

Fig. 6. Ca^{2+}-dependent binding of nitrendipine to calmodulin. Calmodulin (150 μg) was applied to a Sephadex G-25 column (1.0 × 13.2 cm) equilibrated at room temperature with 50 mM MOPS (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10 μM [5-methyl-^{3}H]nitrendipine, 5% DMSO, and either 0.1 mM $CaCl_2$ (●) or 1.0 mM EGTA (○). Eluent fractions of 0.2 ml were collected at a flow rate of 0.2 ml/min and assayed for radioactivity (———) and protein content (— — —).

These results suggested that dihydropyridines might be inhibiting MLCK-catalyzed smooth muscle myosin light chain phosphorylation by binding to calmodulin and blocking the activation of MLCK. We therefore studied the binding of nitrendipine to calmodulin directly by means of molecular sieve chromatography as described in Methods (Fig. 6). Calmodulin was applied to a Sephadex G-25 column equilibrated with various concentrations of tritium-labeled nitrendipine. Eluent fractions were tested for protein concentration and radioactivity. Binding of nitrendipine to calmodulin was determined by measuring the increase in radioactivity in protein-containing fractions. Binding of nitrendipine to calmodulin was found to occur only in the presence of Ca^{2+}. Dihydropyridines are insoluble at concentrations required to saturate calmodulin binding under these conditions. Thus, only high-affinity binding could be characterized, and the number of drug binding sites per molecule of calmodulin could not be determined accurately. The K_{app} for nitrendipine-calmodulin binding, based upon the model of one to one binding and representing the average of nine pairs of nitrendipine and calmodulin concentrations derived from three experiments, was $5.2 \pm 0.3 \times 10^{-5}$ M.

DISCUSSION

Our studies demonstrate the ability of dihydropyridine Ca^{2+} antagonists to inhibit the MLCK-catalyzed phosphorylation of smooth muscle myosin light chains *in vitro*. This inhibition most likely occurs via binding of dihydropyridines to calmodulin, resulting in an inability of calmodulin to activate MLCK, and does not appear to involve inhibition at the active site of the enzyme. These conclusions are based upon the similarity of the K_{app} for binding of nitrendipine to calmodulin to the IC_{50} for inhibition of MLCK activity by the drug, as well as upon the inability of felodipine to inhibit the activity of MLCK from which the calmodulin binding site has been removed. Similar IC_{50} values were noted for the inhibition of calmodulin-activated cyclic nucleotide phosphodiesterase activity by the dihydropyridines nimodipine and nicardipine [13]. No direct study of dihydropyridine–calmodulin binding was attempted, however; furthermore, calmodulin-independent phosphodiesterase activity also was inhibited by the drugs. Thayer and Fairhurst [25], studying nitrendipine–calmodulin interactions by means of equilibrium dialysis, found only low affinity non-saturable binding. Our efforts at equilibrium dialysis studies were confounded by the adherence of dihydropyridines to a wide variety of surfaces, most notably dialysis membranes. We feel that the gel filtration method described herein provides a more accurate measure of drug–protein binding in this system. Our data were insufficient to determine precisely the number of high affinity dihydropyridine binding sites on calmodulin or the stoichiometry required for inhibition of MLCK activity, as the drug was present in large excess relative to calmodulin in the enzyme assay. The possibility that dihydro-

pyridines act by binding to MLCK at the calmodulin-binding site cannot be excluded, but seems improbable in light of the above observations.

Inhibition of MLCK activation by dihydropyridines is not competitive with respect to Ca^{2+}, as increasing the Ca^{2+} concentration failed to overcome the inhibition. Rather, our calmodulin binding studies indicate that inhibition was uncompetitive with respect to Ca^{2+}, in that nitrendipine binding to calmodulin occurred only in the presence of bound Ca^{2+}. Binding of Ca^{2+} to calmodulin exposes hydrophobic sites which are believed to be involved in the activation of calmodulin-dependent enzymes [26]. Dihydropyridines most likely bind to these same hydrophobic sites and thereby inhibit enzyme activation. In this sense the mechanism of dihydropyridine inhibition of MLCK activity is similar to that previously described for other calmodulin antagonists [23, 27, 28]. Further studies to determine more precisely the relation between Ca^{2+} and dihydropyridine binding by calmodulin are in progress.

The physiologic significance of these observations is uncertain. Dihydropyridines exert their effects on Ca^{2+} influx and smooth muscle contraction at concentrations several orders of magnitude lower than their IC_{50} values for inhibition of MLCK activity [29, 30]. There is evidence that dihydropyridines may be concentrated 200- to 500-fold intracellularly [31], however, and the significance of long-term intracellular accumulation of these drugs has yet to be determined. Andersson *et al.* [14], studying drug–calmodulin interactions by means of ^{113}Cd nuclear magnetic resonance spectra, found that the physiologically inactive oxidized form of felodipine causes spectral shifts similar to those caused by the active compound. Inhibition of calmodulin-dependent enzyme activation was not examined, however. Kreye *et al.* [32] found that dihydropyridines failed to inhibit calmodulin-dependent tension development in chemically skinned smooth muscle, while other calmodulin antagonists were inhibitory. We have noted that dihydropyridines at concentrations similar to those used in this study are able to interfere with other intracellular processes which regulate contractility, suggesting that the intracellular actions of these drugs may be quite complex [33].

The mechanisms studied in this paper may provide a model for understanding the interactions of Ca^{2+} antagonists with proteins mediating Ca^{2+} influx across cell membranes. Furthermore, calmodulin mediates a number of Ca^{2+}-dependent reactions involved in muscle contraction, including activation of cyclic nucleotide phosphodiesterase and the phosphorylation of phospholamban in cardiac sarcoplasmic reticulum [8]. Dihydropyridines may therefore prove useful in studying the role played by calmodulin in the Ca^{2+}-mediated regulation of contractility.

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