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The Interaction of Dihydropyridine Calcium Channel Blockers with Calmodulin and Calmodulin Inhibitors

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SUMMARY

The calmodulin inhibitors R24571 and trifluoperazine were found to inhibit competitively the binding of [^{3}H]nitrendipine to a 48,000 × *g* particulate fraction of rat brain with IC_{50} values of 1.0 and 18.8 μM, respectively. Equilibrium dialysis was used to test the ability of the dihydropyridines nitrendipine, felodipine, and nicardipine to inhibit the binding of [^{3}H]chlorpromazine, [^{14}C]pimozide, and $^{45}Ca^{2+}$ to calmodulin. At dihydropyridine concentrations near the limit of solubility (10 μM), the only significant effect in these three binding experiments was a 26% inhibition of [^{14}C]pimozide binding to calmodulin by nicardipine, indicating that the dihydropyridines do not bind to the same site on calmodulin as chlorpromazine, pimozide, or calcium. Equilibrium dialysis was also used to determine the ability of the dihydropyridines to interact directly with calmodulin. [^{3}H] Nitrendipine bound to calmodulin in a calcium-dependent manner; however, this binding was of a low-affinity, unsaturable nature. These results suggest that the dihydropyridine drugs do not interact with calmodulin at concentrations that are pharmacologically significant.

The clinical significance of the dihydropyridine calcium channel blockers (1) as well as recent advances in the study of their membrane binding characteristics (2–4) have led to much interest in the molecular characteristics of the "calcium channel"-dihydropyridine binding site. Membrane binding sites for the dihydropyridine [^{3}H]nitrendipine have been found in ileum, heart, aorta, and brain, and the skeletal muscle sarcoplasmic reticulum (2–6). The ability of the dihydropyridines to block the inward calcium current in mammalian myocardium (7) and the good correlation between membrane binding and inhibition of contractile response in the guinea pig ileum (2) has led to the generally accepted belief that these drugs bind specifically to voltage-dependent calcium channels.

The work of Boström *et al.* (8) implicated calmodulin as playing a role in the action of the dihydropyridines, owing to the ability of felodipine to alter the ^{113}Cd-nuclear magnetic resonance spectrum of calmodulin. Furthermore, Sakamoto *et al.* (9) and Epstein *et al.* (10) implicated calmodulin by reporting the ability of nicardipine and nimodipine to inhibit calmodulin-regulated cyclic AMP phosphodiesterase. More recent reports have described calcium-dependent [^{3}H]nitrendipine binding to brain membranes at calcium concentrations that are sufficient to activate calmodulin (11).

In this study we investigated the possible role of calmodulin at the dihydropyridine binding site. Calmodulin inhibitors were found to inhibit competitively the binding of [^{3}H]nitrendipine to brain membranes, suggesting calmodulin as a possible binding site for the dihydopyridines. [^{3}H]Nitrendipine bound to calmodulin in a calcium-dependent manner. However, the low affinity and unsaturable nature of this binding led to our conclusion that calmodulin is not a pharmacologically relevant dihydropyridine binding site.

[^{3}H]Nitrendipine membrane binding assays were performed basically as described by Ehlert *et al.* (3). Cerebral cortices of male Sprague-Dawley rats were homogenized with a Polytron in 10 volumes of 25 mM Na/Hepes[2] buffer (Calbiochem-Behring) (pH 7.4) and washed three times by centrifugation at 48,000 × *g* for 10 min, followed by resuspension in fresh buffer. This particulate fraction was used either immediately after preparation or after freezing. Binding assays (2 ml total volume) contained [^{3}H]nitrendipine, 12.5 mM Na/Hepes buffer (pH 7.4), approximately 1 mg of protein of brain membranes, and, when appropriate, trifluoperazine or R24571. After a 30-min incubation in the dark at 25°, the assay mixtures were cooled to 0° and filtered through Whatman GF/B filters under vacuum. The filters were washed twice with 10-ml aliquots of ice-cold 12.5 mM Na/

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[2] The abbreviations used are Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; R24571, 1-[bis(*p*-chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl]imidazolium chloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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Hepes buffer (pH 7.4). The filters were then counted in CytoScint (WestChem) scintillation cocktail. Nonspecific binding was defined as [^{3}H]nitrendipine binding in the presence of 1 μM nifedipine, a concentration that produces maximal inhibition of specific binding. IC_{50} and K_D values were calculated as described by Rodbard and Frazier (12) and Scatchard (13), respectively.

Equilibrium dialysis was performed as described by Levin and Weiss (14). Calmodulin, at a concentration of 300 μg/ml for [^{3}H]nitrendipine binding or 100 μg/ml for [^{3}H]chlorpromazine, [^{14}C]pimozide, and $^{45}Ca^{2+}$ binding, was placed in Spectrapore-1 dialysis tubing (M_r cutoff 8000; Spectrum Medical Industries, Inc.) and dialyzed for 16 hr at room temperature, protected from light, with constant stirring in a 50-ml bath containing 12.5 mM Na/Hepes buffer (pH 7.4), 1 mM $MgCl_2$, radiolabeled drugs or $^{45}Ca^{2+}$ (1.07 Ci/mmole, Amersham), inhibitors, and either 0.1 mM $CaCl_2$ or 0.3 mM EGTA. The moles of radioligand bound to calmodulin were determined by subtracting the mean number of counts from two 100-μl aliquots of the bathing medium at the end of the experiment (free radioligand) from the mean of two 100-μl aliquots of the calmodulin solution from inside the dialysis bags. Two or three replicate samples were dialyzed per bath.

Drugs were obtained from the following suppliers: [^{3}H]nitrendipine (87.4 Ci/mmole) and [^{3}H]chlorpromazine (21.0 Ci/mmole) from New England Nuclear Corporation; [^{14}C]pimozide (14.1 mCi/mmole) from McNeil Pharmaceuticals; nicardipine from Yamanouchi Pharmaceutical Company, Ltd.; felodipine from Hassle, Sweden; nitrendipine from Miles Pharmaceuticals; trifluoperazine from Smith Kline & French Laboratories; and R24571 from Janssen Pharmaceuticals. [^{14}C]Pimozide was purified by thin-layer chromatography using silica gel 60 0.2-mm plates (E. Merck) with 5% methanol in chloroform as the solvent. Nifedipine was synthesized in our laboratory.

Bovine brain calmodulin was isolated by the method of Charbonneau and Cormier (15), rendered free from column buffers and EGTA by repeated ultrafiltration and dilution with 25 mM Na/Hepes buffer (pH 7.4), and confirmed pure by analytical 15% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (16). Protein was determined by the method of Lowry *et al.* (17), with bovine serum albumin as the standard.

The control [^{3}H]nitrendipine binding isotherm to brain membranes was in agreement with results previously reported (2–4), with a K_D of 0.19 nM. The specific component of this binding could be inhibited by drugs known to interfere with calmodulin function. The binding of 0.3 nM [^{3}H]nitrendipine was found to be inhibited by trifluoperazine and R24571 in a dose-dependent manner (Fig. 1). Trifluoperazine is a phenothiazine known to bind to and inhibit some of the functions of calmodulin (18). Similarly, R24571 has been reported to be more potent than trifluoperazine as an inhibitor of the calmodulin-regulated enzymes, brain phosphodiesterase, erythrocyte Ca^{2+}-ATPase, and phosphorylase *b* kinase (19). The results presented here indicate that R24571 is approximately 19 times more potent than trifluoperazine, with IC_{50} values equal to 1.0 and 18.8 μM, respectively.

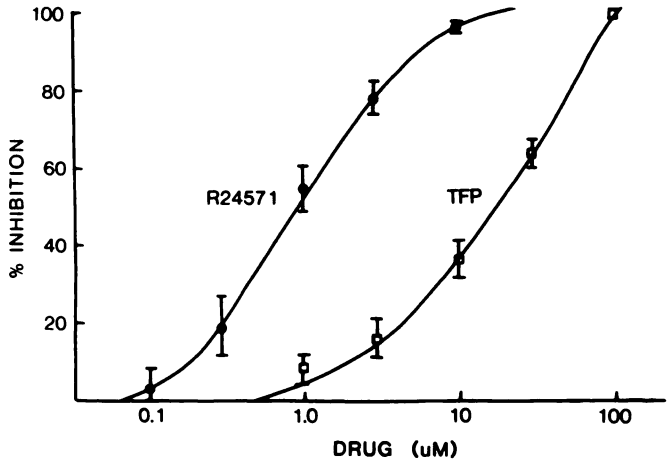

FIG. 1. *Inhibition of [^{3}H]nitrendipine binding by calmodulin antagonists*

The inhibition of 0.3 nM [^{3}H]nitrendipine binding to rat brain membranes by R24571 (●) and trifluoperazine (*TFP*) (□). The data are means ± standard error of three triplicate experiments.

To characterize the nature of the competition between [^{3}H]nitrendipine and calmodulin inhibitions, [^{3}H]nitrendipine binding isotherms were generated at R24751 concentrations of 0, 0.1, 0.3, and 1.0 μM. These data are displayed as a Scatchard plot in Fig. 2. Curves were fitted by linear regression analysis, and the *x*-intercepts (B_{max}) were not significantly different at a 95% confidence limit. The decreasing slope (increasing K_D) of the Scatchard plots in the presence of increasing inhibitor concentrations, coupled with a constant B_{max}, clearly classifies

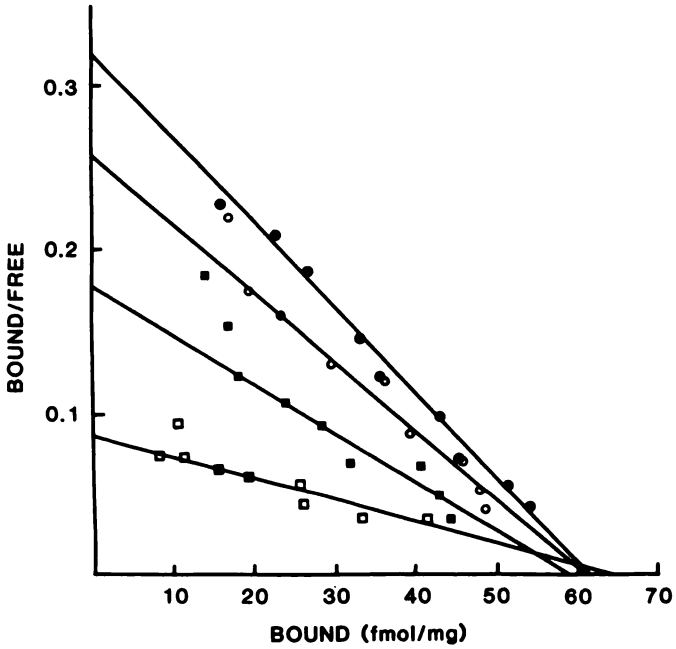

FIG. 2. *Scatchard analysis of the effects of R24571 on [^{3}H]nitrendipine binding*

A representative analysis of [^{3}H]nitrendipine binding to rat brain membranes in the absence (●) and presence of 0.1 μM R24571 (○), 0.3 μM R24571 (■), and 1.0 μM R24571 (□). [^{3}H]Nitrendipine concentrations ranged from 0.08 nM to 1.28 nM.

R24571 as a competitive inhibitor of [^{3}H]nitrendipine binding. These data suggested that the calmodulin inhibitors and the dihydropyridines might have a common binding site in brain.

The possibility that calmodulin may be a common binding site shared by the calmodulin inhibitors and the dihydropyridines was investigated by assaying directly the interaction between the dihydropyridine calcium channel blockers and calmodulin by equilibrium dialysis. Calmodulin, 0.3 mg/ml, was dialyzed against various [^{3}H] nitrendipine concentrations as described above. To obtain the higher nitrendipine concentrations, the high specific activity [^{3}H]nitrendipine was diluted with unlabeled nitrendipine. No [^{3}H]nitrendipine was bound to calmodulin in the presence of 0.3 mM EGTA. However, in the presence of 0.1 mM Ca^{2+}, [^{3}H]nitrendipine bound to calmodulin. This binding was not saturable, as indicated by the linear isotherm displayed in Fig. 3. Linear regression analysis, after taking the logarithm of the data points, fit a straight line to these data with a correlation coefficient of 0.998. Because this binding was not saturable, no affinity constant could be derived. However, at an equivalent concentration of 1.0 μM, 240 times more [^{14}C]pimozide than [^{3}H]nitrendipine binds to calmodulin, suggesting a very low affinity binding of [^{3}H]nitrendipine to calmodulin.

The dihydropyridines nicardipine, felodipine, and nitrendipine also were tested for their abilities to inhibit the binding of [^{3}H]chlorpromazine, [^{14}C]pimozide, and $^{45}Ca^{2+}$ to calmodulin. Both [^{3}H]chlorpromazine and [^{14}C] pimozide bound to calmodulin in the calcium-dependent manner described by Levin and Weiss (20), but only nicardipine showed any significant inhibitory effect in three separate experiments. Nicardipine, at concentrations approaching its solubility limit (10 μM), inhibited [^{14}C]pimozide binding by 26% at a free pimozide concentration of 0.64 μM. $^{45}Ca^{2+}$ was found to bind to calmodulin as previously described (21), but no inhibitory effects of any of the three compounds could be found.

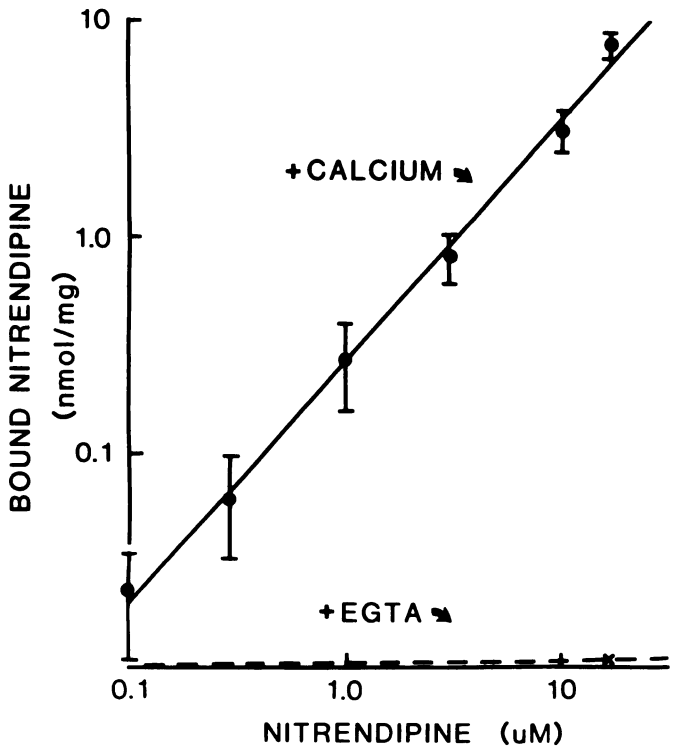

FIG. 3. *Calcium-dependent [^{3}H]nitrendipine binding to calmodulin*

Equilibrium dialysis was performed in 50-ml baths containing 12.5 mM Na/Hepes buffer (pH 7.4), 1 mM $MgCl_2$, [^{3}H]nitrendipine, and either 0.1 mM $CaCl_2$ (●) or 0.3 mM EGTA (×). Samples (0.3 ml) of calmodulin (300 μg/ml) were dialyzed for 16 hr. Each point represents the mean ± standard error of three samples. Note that each axis is presented on a logarithmic scale.

The competitive inhibition of [^{3}H]nitrendipine binding to brain membranes by the calmodulin inhibitor, R24571, supported our initial hypothesis that calmodulin plays a role at the "calcium channel"-dihydropyridine binding site while at the same time placed a number of constraints upon the type of interaction that may occur. This type of interaction eliminated nonspecific membrane effects as a possible mechanism of the inhibition. Additionally, the possible role of calmodulin in dihydropyridine binding was limited to either being permanently coupled via an allosteric linkage to the binding site or being the actual site of binding. The concept of calmodulin's being the dihydropyridine binding site was supported by our finding that [^{3}H]nitrendipine binding was inhibited competitively by calmodulin inhibitors and by the reports of other workers describing dihydropyridine-induced changes in calmodulin's ^{113}Cd-nuclear magnetic resonance spectrum, dihydropyridine inhibition of calmodulin-regulated cyclic AMP phosphodiesterase, and the calcium requirement for [^{3}H]nitrendipine binding to brain membranes (8–11). Therefore, we investigated a direct binding of the dihydropyridines to calmodulin by equilibrium dialysis. While [^{3}H]nitrendipine bound to calmodulin in a calcium-dependent manner, the unsaturable, low-affinity nature of this binding led us to conclude that it is merely a hydrophobic interaction previously described for many nonpolar compounds with calmodulin (22). The ability of nicardipine to inhibit weakly [^{14}C]pimozide binding may account for the inhibition of cyclic AMP phosphodiesterase found by others (9–10). The inability of felodipine to inhibit any of the calmodulin systems tested suggests that the millimolar concentrations at which the work on ^{113}Cd-nuclear magnetic resonance spectrum changes was carried out (8) were too high to be pharmacologically relevant. We have not eliminated the unlikely possibilities that calmodulin is linked to the dihydropyridine binding site by means of a nondissociable linkage that allosterically interacts with the dihydropyridine binding site, or that calmodulin needs a specific membrane environment for the binding of the dihydropyridines. However, the weak interaction of [^{3}H]nitrendipine with calmodulin suggests that dihydropyridine drugs do not interact directly with calmodulin at the nanomolar concentrations at which they are pharmacologically active.

In seeking an explanation for the interaction of the calmodulin antagonists with [^{3}H]nitrendipine binding to brain, it seems reasonable to conclude that the calcium-dependent [^{3}H]nitrendipine binding sites in rat brain membranes possess some similarities to the calcium-de-

pendent antipsychotic drug-binding sites on calmodulin. Finding that calmodulin inhibitors may interact at the calcium channel suggests an additional mechanism to explain some of the pharmacological effects of the antipsychotic drugs, particularly their ability to inhibit contraction in smooth muscle (23).

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