Binding of a Calcium Antagonist, [^{3}H]Nitrendipine, to High Affinity Sites in Bovine Aortic Smooth Muscle and Canine Cardiac Membranes

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ABSTRACT [^{3}H]Nitrendipine, a potent calcium channel antagonist [3-ethyl-5-methyl-1-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine carboxylate], was used to label high affinity binding sites on membranes prepared from bovine aortic smooth muscle. The binding of [^{3}H]nitrendipine is rapid ($t_{1/2}$ < 5 min) and reversible at 37°C. The binding sites have a high affinity for [^{3}H]nitrendipine with an equilibrium dissociation constant of 2.1 nM. The density of sites is 40–60 fmol/mg of membrane protein. Analogues of nitrendipine compete for the binding sites with affinities consistent with their known biological effects as calcium antagonists. Nisoldipine, [isobutyl methyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine carboxylate], a calcium antagonist more potent than nifedipine [2,6-dimethyl-3,5-dicarbo methoxy-4-(2-nitrophenyl)-1,4-dihydropyridine] in relaxing vascular smooth muscle, has an affinity threefold higher than that of nifedipine in competing for the binding sites. A biologically inactive derivative of nifedipine does not compete for [^{3}H]nitrendipine binding. Verapamil (α-isopropyl-α[(*N*-methyl-*N*-homoveratryl)-α-aminopropyl]-3,4-dimethyoxyphenyl acetonitrile), a structurally different calcium antagonist, only partially (25%) inhibits binding at high concentrations (1 μM). Prazosin, an alpha adrenergic antagonist does not compete for [^{3}H]nitrendipine binding sites. The binding of [^{3}H]nitrendipine is not affected by 1.5 mM calcium. Canine cardiac membranes also have high affinity [^{3}H]nitrendipine binding sites, (K_D = 6 nM) but bovine erythrocytes do not. The relative affinities of nisoldipine and nifedipine for the cardiac membrane binding sites reflect the relative activities of these compounds as calcium channel antagonists. These results suggest that the [^{3}H]nitrendipine binding sites are the sites through which dihydropyridines act as calcium channel antagonists.

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INTRODUCTION

The regulation of cellular calcium influx through voltage-sensitive calcium channels is important for the contractile and metabolic functions of smooth muscle. Little is known about the molecular nature of the calcium channel because methods for identifying the channel in cell-free systems have not been available. By contrast, voltage-sensitive sodium channel proteins have been extensively studied by using specific sodium channel blockers such as tetrodotoxin to identify and isolate the regulatory components of the channels (1).

Recently, Fleckenstein (2) described a new class of compounds that appear to block calcium influx in smooth muscle and cardiac tissue, presumably by interacting directly with the calcium channel. One of these compounds, nifedipine, is particularly potent and selective in its blockade of processes thought to be mediated by voltage-sensitive calcium channels. For example, nifedipine blocks the slow inward current of the action potential (3), inhibits cardiac contractility (4), prevents smooth muscle contraction induced by depolarization (2), and blocks the influx of Ca^{2+} into smooth muscle during contraction (5). Because of these specific calcium channel blocking effects, nifedipine is an extraordinarily useful clinical vasodilator (6).

There is little information available on the molecular mechanism by which nifedipine interacts with the plasma membrane to inhibit calcium channels. Because it has highly specific biological effects at very low concentrations (<1 nM), it is likely that nifedipine acts by first binding to a distinct high affinity mac-

romolecular structure, possibly the calcium channel. In this publication we report that a radioactively labeled analogue of nifedipine, [^{3}H]nitrendipine (Fig. 1), can be used to label high affinity binding sites in bovine smooth muscle cell membranes. These experiments have shown that the binding site has the characteristics of the site through which nifedipine acts as a calcium channel blocker. The possibility that this site is a component of the voltage-sensitive calcium channel is discussed.

METHODS

Membrane preparation. The media of bovine aortas was separated from intima and adventitia. Strips of media were minced into 1–2-mm cubes, which were homogenized in 3 vol of 0.25 M sucrose, 10 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ by three 10-s bursts with a Brinkmann polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) set at the highest speed. The mixture was then filtered through a double layer of cheese cloth followed by filtration through nylon screen. The filtrate was centrifuged at 40 *g* for 10 min to remove large particles, and the pellet was discarded. The supernatant was centrifuged at 30,000 *g* for 10 min, and the resulting pellet was washed twice in incubation buffer (150 mM NaCl, 10 mM Tris–HCl (pH 7.5) and resuspended in this buffer at a final protein concentration of 4 mg/ml. Erythrocyte membranes were prepared by lysing washed bovine erythrocytes (collected in citrated tubes) in 5 mM Tris and 5 mM $MgCl_2$ followed by homogenization in a Potter-Elvejhm homogenizer. Cardiac membranes were prepared by applying the same procedures described above for smooth muscle membranes to minces of canine cardiac tissue obtained from right and left ventricles.

Binding studies. 100 μl of membrane suspension in incubation buffer was added to 20 μl of a fresh aqueous solution of [^{3}H]nitrendipine and 20 μl of appropriate unlabeled compound or water. The final concentration of [^{3}H]nitrendipine was 2 nM unless specified otherwise. The incubation (140 μl) was continued for 10 to 15 min at 37°C with gentle shaking in near darkness. The incubation was terminated by diluting 110 μl of the incubation mixture into 7.5 ml of ice-cold incubation buffer that was poured over a Whatman GFC filter (Whatman Inc., Paper Div., Clifton, NJ) attached to a vacuum. The filter was washed for 10 min with 7.5 ml of incubation buffer and was dried and counted in a liquid scintillation counter at an efficiency of 35%. Nonspecific binding was estimated as the amount of binding of [^{3}H]nitrendipine measured in the presence of 0.1 μM or 1 μM nifedipine. In over 30 experiments the nonspecific binding was usually ~30% of the total amount of binding when the concentration of [^{3}H]nitrendipine was 2 nM.

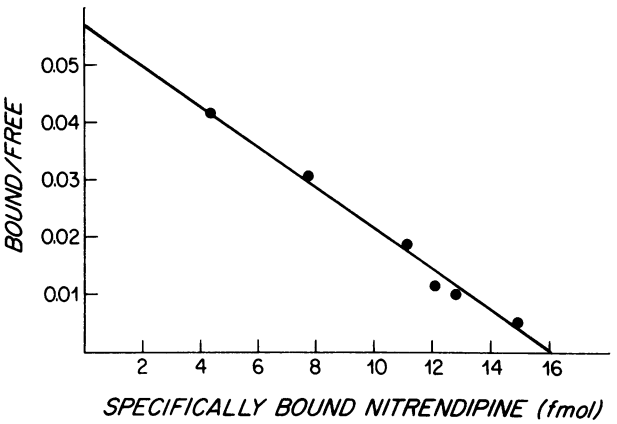

FIGURE 2 Scatchard plot of [^{3}H]nitrendipine (0.5–20 nM) was incubated with bovine aortic membranes for 12 min at 37°C. Nonspecific binding (in the presence of 0.1 μM nifedipine) was deducted from the total binding. The protein concentration was 3 mg/ml in a volume of 140 μl. Each point represents the mean of two determinations. The line was drawn by linear regression analysis (r = 0.99). The slope was used to compute the dissociation constant (2.1 nM). The intercept with the abscissa was used to compute the density of sites (40 fmol/mg protein). This experiment is representative of three separate experiments that gave values of 40, 56, and 63 fmol/mg protein, respectively.

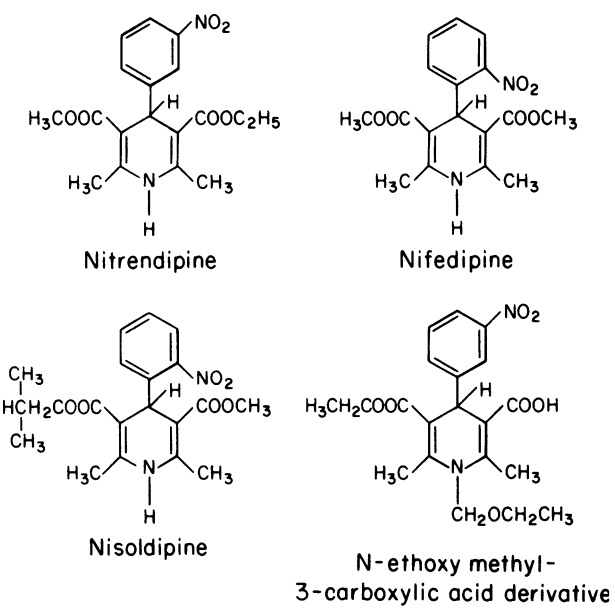

FIGURE 1 Structures of some dihydropyridine derivatives.

Compounds. [^{3}H]Nitrendipine (sp act, 86 Ci/mmol), tritiated by New England Nuclear (Boston, MA), > 99% pure as assessed by thin-layer chromatography on silica gel plates in chloroform/acetone (95:5) and hexane/acetone (70:30). *N*-Ethoxymethyl-5-ethoxycarbonyl-2,6-dimethyl-4-(meta nitrophenyl)-1,4-dihydropyridine-3-carboxylic acid was also obtained from New England Nuclear. Nitrendipine, nisoldipine, and nimodipine [(isopropyl 2-methoxy-ethyl)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinecarboxylate] were obtained from Miles Laboratories Inc., Elkhart, IN.

RESULTS

[^{3}H]Nitrendipine, a potent calcium antagonist (7) (Fig. 1), bound to a finite number of high affinity sites in vascular smooth muscle membranes (Fig. 2). Scatchard analysis (Fig. 2) showed a single order of sites with an equilibrium dissociation constant of 2.1 nM and a density of 40 fmol/mg of protein in these crude membranes from bovine aortic smooth muscle. The binding

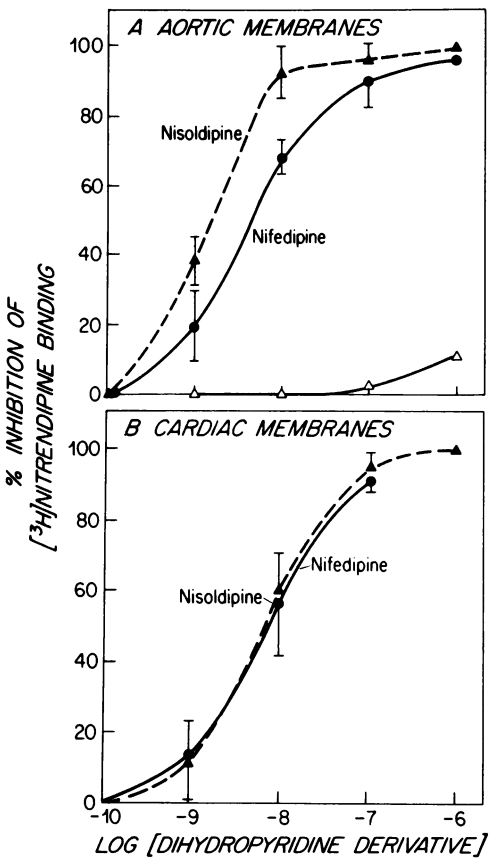

FIGURE 3 Competition of dihydropyridine derivatives for [^{3}H]nitrendipine binding sites. Bovine aortic membranes (A) or canine cardiac membranes (B) were incubated with the indicated concentrations of nisoldipine (—▲—), nifedipine (—●—), or 1-ethoxymethyl-5-ethoxy carbonyl-2,6-dimethyl-4-(metanitrophenyl)-1,4 dihydropyridine-3-COOH (—△—) in the presence of 3–4 nM [^{3}H]nitrendipine and specific binding was determined as described in Methods. Each point represents the mean±SD of values from duplicate incubations in five(nisoldipine) or seven(nifedipine) separate experiments. The values for the carboxylic acid derivate represent two experiments.

of [^{3}H]nitrendipine (2 nM) was rapid, attaining steady state within 3 min, and was reversible with a half-time of dissociation of 2–4 min at 37°C (not shown). At 4°C the rate of dissociation was very slow (<15% dissociation in 10 min), thus validating the use of the filtration assay method used in this work.

The specificity of the binding sites (Fig. 3, Table I) reflected the relative abilities of dihyropyridines to block calcium channels in vascular smooth muscle. Nisoldipine had the highest affinity for the binding sites, causing half-maximal inhibition of [^{3}H]nitrendipine binding at a concentration (1.5 nM) that is similar to the concentration of (2.4 nM) of nisoldipine, which half-maximally inhibits the contraction of vascular smooth muscle (8). Nifedipine was less potent, requiring a concentration of 4.0 nM to block half of the binding sites, again agreeing with the reported IC_{50} of nifedipine (8.1 nM) in blocking vascular smooth muscle contraction (8). The *N*-ethoxy methyl-3-carboxylic acid derivative (Fig. 1, Table I) did not inhibit binding at a concentration of 1,000 nM, consistent with the observation that carboxylic acid and *N*-substituted derivates of dihydropyridines do not block calcium channels (10). Verapamil, a calcium channel antagonist that is less potent and less specific than nifedipine (10), inhibited binding by only 25% at a concentration of 1 μM and caused little or no inhibition at lower concentrations. This is consistent with the observation that verapamil blocks slow inward current and smooth muscle contraction in a manner that is qualitatively different from the blockade induced by nifedipine, suggesting that these structurally dissimilar compounds have different sites of action. The alpha adrenergic antagonist prazosin did not inhibit [^{3}H]nitrendipine binding in concentrations up to 1 μM. The presence of 1.5 mM calcium did not affect the binding of [^{3}H]nitrendipine.

Cardiac membranes bound [^{3}H]nitrendipine with high affinity (K_D = 6 nM) consistent with the known effects of dihydropyridines on cardiac calcium channels (3). A higher concentration of nisoldipine (7.0 nM) was required to half-maximally inhibit [^{3}H]nitrendipine binding in cardiac membranes than that required (1.5 nM) in smooth muscle membranes (Table I). This is consistent with the observation that nisoldipine is more potent in blocking calcium channels in vascular tissue than cardiac tissue (9). It has been proposed (9) that

TABLE I
Comparison of Binding Affinities in Cardiac and Vascular Tissue

	Aorta binding IC_{50}	Cardiac binding IC_{50}
	nM	
Nisoldipine	1.5	7.0
Nitrendipine	2.0	6.4
Nimodipine	1.8	—
Nifedipine	4.1	7.9
N-Ethoxy methyl-3-carboxylic acid derivative	>1,000	—
Prazosin	>1,000	>1,000
Verapamil	>1,000*	>1,000*

"Binding IC_{50}" refers to the concentration of compound that inhibits half of the specific binding of 2 nM [^{3}H]nitrendipine to aorta smooth muscle membranes or to cardiac ventricular membranes.
* A high concentration (1 μM) of verapamil caused only 25% inhibition of binding in aortic membranes and 30% inhibition in cardiac membranes. Prazosin (1 μM) caused no inhibition of binding.

the relative selectivity of nisoldipine for vascular rather than cardiac tissue might make it a more efficacious vasodilator than nifedipine, which is less selective. Thus, nifedipine and nisoldipine were equipotent in competing for cardiac [^{3}H]nitrendipine binding sites, whereas nisoldipine was significantly more potent than nifedipine in competing for aortic binding sites (Fig. 3, Table I).

Unlike vascular and cardiac membranes, bovine erythrocyte membranes had no detectable specific binding sites for [^{3}H]nitrendipine.

DISCUSSION

Our data demonstrate that nitrendipine, a potent calcium channel antagonist (7), binds to saturable high affinity sites in smooth muscle and cardiac ventricular membranes. The density of [^{3}H]nitrendipine binding sites (40–60 fmol/mg membrane protein) is comparable to the density of many hormone receptors (11). The tissue distribution of these binding sites, the high affinity of binding, and the agreement of the binding affinities with the reported affinities of dihydropyridines as calcium antagonists, make it likely that these binding sites are the sites through which these compounds act as vasodilators and negative inotropic agents. Whether the sites are directly on the macromolecules that compose the calcium channel or are on separate molecules that regulate calcium-mediated phenomena will require further investigation.

It is not surprising that verapamil, a calcium channel antagonist structurally different from nifedipine, is not a potent competitor for [^{3}H]nitrendipine binding sites because the pharmacological characteristics of verapamil and nifedipine differ greatly (10). Unlike nifedipine, verapamil requires a much longer incubation and high concentrations to achieve a maximal effect; it nonspecifically blocks the fast channel as well as the slow channel, and its action is frequency dependent. These observations have led to the proposal that verapamil may act like a local anesthetic, possibly on the inside surface of the sarcolemma, whereas nifedipine may act directly on the calcium channel in a manner analogous to the action of tetrodotoxin on the sodium channel (10). Much more work is yet to be done on the subcellular distribution, regulation, purification, and characterization of nitrendipine binding sites. The binding studies described here provide a method for investigating the mechanism of action of this important class of compounds and may be useful in future studies of the biochemistry of voltage-sensitive calcium channels.

Note added in proof. Following submission of this manuscript the following publications demonstrating [^{3}H]nitrendipine binding sites in cardiac tissue have appeared: Ehlert et al. 1982. *Biochem. Biophys. Res. Comm.* **104:** 937–943; Bellemann, P. et al. 1981. *Arzneim. Forsch.* **31**(II): 2064–2065; Murphy, K. M. M., and S. H. Snyder. 1982. *Eur. J. Pharmacol.* **77:** 201–202; Bolger et al. 1982. *Biochem. Biophys. Res. Comm.* **104:** 1604–1608.

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