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SPECIFIC BINDING OF [^{3}H]NITRENDIPINE TO MEMBRANES FROM CORONARY ARTERIES AND HEART IN RELATION TO PHARMACOLOGICAL EFFECTS. PARADOXICAL STIMULATION BY DILTIAZEM

A. DePover, M.A. Matlib, S.W. Lee, G.P. Dubé, I.L. Grupp, G. Grupp, and A. Schwartz*

Departments of Pharmacology and Cell Biophysics and Physiology and Medicine (Division of Cardiology) University of Cincinnati, College of Medicine Cincinnati, OH 45267

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SUMMARY: High affinity binding sites for the calcium channel inhibitor [^{3}H]nitrendipine have been identified in microsomes from pig coronary arteries (K_D=1.6 nM; B_{max}=35 fmol/mg) and in purified sarcolemma from dog heart (K_D=0.11 nM; B_{max}=230 fmol/mg). [^{3}H]nitrendipine binding to coronary artery microsomes was completely inhibited by nifedipine, partially by verapamil and D600 and, surprisingly, was stimulated by d-cis-diltiazem but not by l-cis-diltiazem, a less active isomer. Half-maximal relaxation of KCl-depolarized coronary rings occurred in a slow process at 1 nM nitrendipine or 100 nM d-cis-diltiazem. In dog trabecular strips, nitrendipine caused a negative inotropic response (ED_{50}= 1μM). These results suggest that there may be multiple binding sites for different "subclasses" of calcium channel inhibitors, and that drug binding sites may be different molecular entities from the putative calcium channels.

INTRODUCTION: The contraction of vascular smooth muscle induced by KCl is blocked by compounds, such as nifedipine, verapamil, diltiazem and cinnarizine, which are thought to specifically block calcium entry into the cell (1-3). The considerable structural diversity of these compounds, however, suggests the existence of different sites of action on the cell membrane. Recently, [^{3}H]nitrendipine has been used to label dihydropyridine receptors in homogenates or membrane preparations from heart, brain, and ileum (4-8), and it has been suggested (6,8) that allosteric interactions occur between the binding sites of nitrendipine and verapamil or D600. Dihydropyridine derivatives, verapamil and diltiazem are powerful coronary vasodilators. The sensitivity of different organs and tissues to these drugs varies considerably with, e.g., the nifedipine-type drug being highly active

*To whom all correspondence should be sent.

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on peripheral vasculature, coronaries and heart, and diltiazem being more selective on the coronaries (3,9-11). For this reason, we have studied [^{3}H]nitrendipine binding to membrane preparations from coronary arteries and from heart.

METHODS: Isolation of coronary artery microsomes. The method was essentially as described by Matlib et al. (12). Fifteen pig hearts were obtained from Kahn's and Company, Cincinnati, Ohio, obtained soon after the animals were sacrificed, placed in ice-cold 0.9% NaCl, and immediately transported to the laboratory. The left anterior descending artery and right circumflex arteries were dissected from the heart. Fat and myocardium were trimmed from the arteries, which were then opened longitudinally with a pair of scissors and placed in ice-cold solution containing 0.25 M sucrose, 10 mM Mops, pH 7.4. All the remaining fat from the outer surface of the artery was scraped off. From the inner surface, the endothelial layer was also scraped off. The cleaned tissue (approximately 6-7 g) was placed in fresh ice-cold sucrose solution. The tissue was homogenized twice by a Polytron (PT 10, Brinkman Instruments Inc.) at a setting of 8 for 10 s, and then once at a setting of 6.5 for 10 s, with 2-3 min time interval between each pass for cooling of the sample. The sample was further homogenized by making 3 passes with a teflon pestle and glass homogenizer (size C, Thomas Corp.) at 1500 rpm. All the homogenized samples were combined by passing through double layer cheesecloth into a plastic beaker. The homogenate was centrifuged at 500 xg for 10 min in a Beckman model J21-C centrifuge fitted with a JA20 rotor. The supernatant was decanted through double layer cheesecloth and centrifuged at 12000 xg for 10 min. The supernatant was centrifuged at 105000 xg for 30 min in a Beckman model L5-50 ultracentrifuge. The resulting pellet (about 6 mg protein) was resuspended in a small volume of 20 mM Mops at pH 7.0. The protein was determined according to Lowry et al.(13).

Isolation of cardiac muscle sarcolemma. Dog heart sarcolemma was prepared according to Van Alstyne et al.(14).

[^{3}H]Nitrendipine binding assay. Freshly prepared microsomes (0.15-0.25 mg) from pig coronary arteries were incubated for 2-30 min at 37°C in 0.1 ml medium containing 50 mM Tris-HCl (pH 7.4) and various concentrations of [^{3}H]nitrendipine (85 Ci/mmol). The binding was stopped by addition of 5 ml of ice-cold, double-distilled water and filtration on Whatmann GF/F glass fiber filters. The filters were washed four times with 5 ml of ice-cold water and placed in scintillation vials containing 10 ml of scintillation solution (Budget-Solve, Research Product International). The binding was proportional to amount of protein in the range of 0.05-0.25 mg. The binding in the presence of 10^{-6} M unlabelled nitrendipine or nifedipine was defined as non-specific (see Fig. 1). The same procedure was used for dog heart sarcolemma, except that 0.025 mg of sarcolemma protein was incubated at 30°C for 30 min. We should like to emphasize that after three months of experiments, thin-layer chromatography (95% chloroform, 5% acetone) revealed the presence of tritiated contaminants (more than 2% of total radioactivity) in some samples of [^{3}H]nitrendipine stock solution. Experiments with these samples gave dissociation constants 10-fold (coronary arteries) or 50-fold (heart) higher than that obtained before, and the binding was not stimulated by d-cis-diltiazem. Further experiments are required to identify the contaminants. The data reported in this paper, however, were obtained with the "uncontaminated" [^{3}H]nitrendipine and were the most reproducible.

Other binding assays. Essentially, the same procedure was used for the other radioligand assays. The incubation medium was supplemented with 5 mM $MgCl_2$ for [^{3}H]dihydroalprenolol (35 Ci/mmol) and [^{3}H]quinuclidinyl benzoate (67 Ci/mmol), 5 mM $MgCl_2$ and 5 mM Tris-phosphate for [^{3}H]ouabain (17 Ci/mmol).

Temperature and incubation time were respectively for the three compounds: 30°C and 30 min, 37°C and 60 min, 37°C and 30 min. The binding in the presence of respectively 10^{-4}M alprenolol, 10^{-6}M atropine, and 10^{-3}M ouabain was defined as non-specific.

Measures of mechanical tension of porcine coronary arteries. A section (4-5 cm) of the right circumflex coronary artery near the aorta was dissected from the heart. Loose adipose and connective tissue were removed from the adventitia prior to transverse segmentation of the artery into rings having a width of 4 mm. The rings were suspended in an organ bath under a resting tension of 3.5 g and were allowed to equilibrate for 2 h in the physiological solution containing 126.9 mM NaCl, 4.7 mM KCl, 1.6 mM $CaCl_2$, 17.7 mM $MgSO_4$, 1.18 mM KH_2PO_4, 18 mM $NaHCO_3$, and 5.5 mM glucose. The solution was saturated with 95% O_2 and 5% CO_2 at 37°C (pH 7.3). Recordings of the contractile activity were made by an isometric force transducer. To test for tissue viability and reproducibility of contraction amplitudes, the rings were challenged with 35 mM KCl several times in succession, rinsing with normal physiological solution after each challenge.

Measures of mechanical tension of cardiac tissue. The mechanical tension of electrically-driven dog trabecular strips was measured according to Grupp et al.(16).

Chemicals. All experiments with dihydropyridines were performed under sodium light. [^{3}H]nitrendipine, [^{3}H]dihydroalprenolol, [^{3}H]quinuclidinyl benzoate, and [^{3}H]ouabain were purchased from New England Nuclear, Boston, Massachusetts. Diltiazem was a gift of Marion Research Laboratories (Kansas City, Missouri), verapamil and D600, of Knoll Pharmaceutical Company (Whippany, New Jersey), and nifedipine, of Pfizer Pharmaceuticals (New York). Nitrendipine and nifedipine were dissolved in ethanol to make 1 mM stock solutions.

RESULTS AND DISCUSSION: The binding of [^{3}H]nitrendipine to porcine coronary artery microsomes was a relatively fast process, steady-state binding being achieved in 10 min (data not shown). When unlabelled nitrendipine or nifedipine was added at steady-state to "chase" the label, the specific binding was rapidly dissociated ($t_{1/2}$ under 2 min). Equilibrium binding (Fig. 1) plotted according to Scatchard (16), revealed a straight line. The number of [^{3}H]nitrendipine binding sites (B_{max}) was much lower in coronary artery microsomes than in cardiac sarcolemma (Table 1) and than that reported for various preparations from heart, brain, and ileum (4-8). This number, however, was the same magnitude as the number of β-adrenergic or muscarinic receptors (Table 1). The dissociation constants also differed between coronary artery microsomes (K_D=1.6 nM) and cardiac sarcolemma (K_D=0.11 nM), but are consistent with the values previously reported in other studies showing tissue variations (4-8).

The specific binding of [^{3}H]nitrendipine (0.6 nM) to coronary artery microsomes was completely inhibited by nifedipine (IC_{50}=8 nM) and partly inhi-

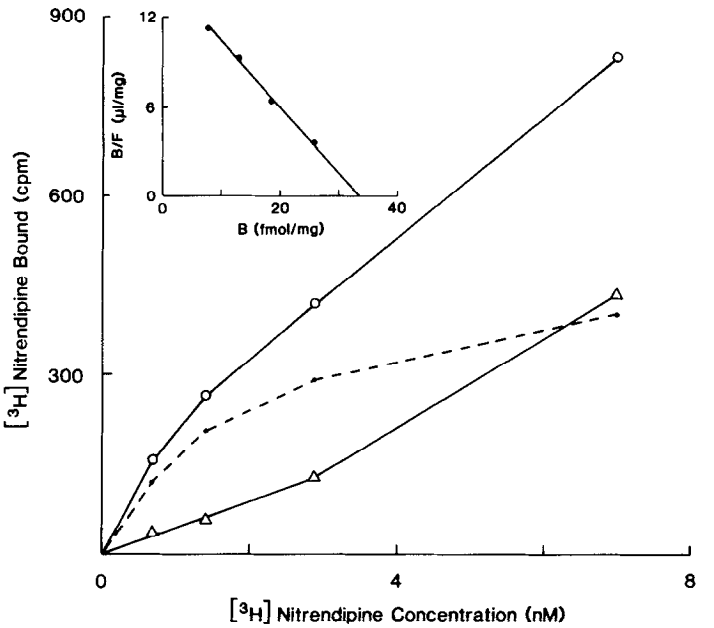

FIGURE 1. Binding of [^{3}H]nitrendipine to microsomes from pig coronary arteries as a function of [^{3}H]nitrendipine concentrations. The data represent a typical experiment: 0.224 mg of microsomal protein was incubated for 10 min at 37°C in the presence of various concentrations of [^{3}H]nitrendipine and in the absence (○) or the presence (△) of 10^{-6} M unlabelled nitrendipine. The dotted line represents specific binding. The points are means of triplicate determinations.
Inset: the specific binding is plotted according to Scatchard.

bited by verapamil and D600 (respectively 62% and 50% at 10^{-6} M). We were surprised to find, however, that the specific binding of [^{3}H]nitrendipine was stimulated by d-cis-diltiazem between 10 and 1000 nM but not by l-cis-diltiazem, a less active isomer (11), (Fig. 2). Ehlert et al. have recently shown an inhibitory effect of diltiazem at 0°C, to heart or brain homogenates, and the absence of any inhibitory effect at 25°C (8). It is possible that the qualitatively different effects of d-cis-diltiazem in our study is due to the higher temperature (37°C). Such an effect of temperature might be related to the fluidity of membrane lipids (17). A stimulation of [^{3}H]nitrendipine binding by d-cis-diltiazem has also been observed in guinea pig ileal microsomes (D.J. Triggle, personal communication) and in guinea pig cardiac microsomes (H. Glossmann, personal communication). This observation suggests the existence of an allosteric interaction between the binding sites of [^{3}H]nitrendipine and

TABLE 1 Binding parameters of nitrendipine and of plasma membrane markers to membranes from pig coronary arteries and dog ventricular muscle. The dissociation constant (K_D) and the maximal binding (B_{max}) were estimated from Scatchard plots by linear regression analysis, in several membrane preparations (N). S.E.M. is indicated where relevant.

Tissue	[^{3}H]Ligand	Temperature (°C)	N	K_D (nM)	B_{max} (fmol/mg)
Pig coronary arteries (microsomes)	Nitrendipine	37	3	1.6 ± 0.5	35 ± 2
	Dihydroalprenolol	30	3	0.7 ± 0.3	40 ± 8
	Quinuclidinyl benzoate	37	2	1.4 ± 0.9	80 ± 7
Dog ventricular muscle (sarcolemma)	Nitrendipine	30	3	0.11 ± 0.01	230 ± 10
	Dihydroalprenolol	30	3	6.5 ± 0.9	2,700 ± 640
	Quinuclidinyl benzoate	37	1	0.15	21,000
	Ouabain	37	3	32 ± 6	370,000 ± 34000

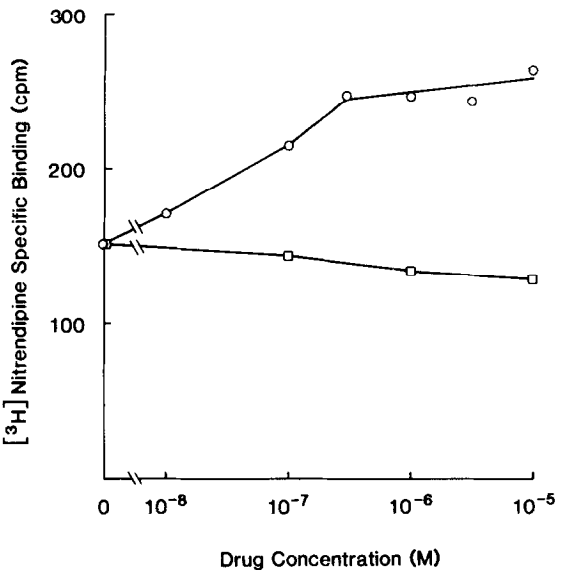

FIGURE 2. Effect of d-cis-diltiazem (O) and l-cis-diltiazem (□) on [^{3}H]nitrendipine binding to microsomes from pig coronary arteries. The data represent a typical experiment: 0.154 mg of microsomal protein was incubated for 15 min at 37°C in the presence of 0.6 nM [^{3}H]nitrendipine and of various drug concentrations. The non-specific binding (49 cpm) was subtracted from the total. 12.7 fmol [^{3}H]nitrendipine per mg protein was specifically bound in the absence of drug. The points are means of triplicate determinations. Similar data were obtained with two other microsomal preparations.

d-cis-diltiazem, which may have important therapeutic implications if it occurs in the pharmacologically active concentration range (see below).

In pharmacological experiments (Fig. 3), nitrendipine or d-cis-diltiazem relaxed coronary artery rings in a dose-dependent manner with half-maximal inhibition of tension occurring at 1.1 nM nitrendipine or 100 nM d-cis-diltiazem. The maximal relaxation by the drugs was about 8% below the initial resting tension. This could be due to a change of the tissue compliance during incubation since the time to obtain maximal response development for any given dose of drug usually extended 1-3 hours (nitrendipine) or 20-40 min (d-cis-diltiazem). The dose-response curve for both these drugs is characteristic of the Langmuir adsorption isotherm curve for a single class of sites. Of considerable interest is the fact that the specific binding of [^{3}H]nitrendipine and the stimulatory effect of d-cis-diltiazem on this binding occur in a pharmacological concentration range. In contrast, in electrically-

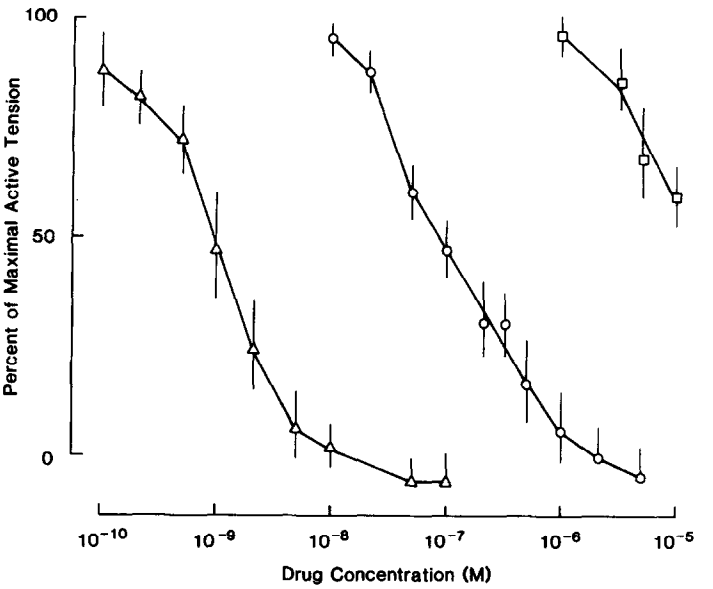

FIGURE 3. Drug-induced relaxation of depolarized pig coronary arteries. Cumulative doses of nitrendipine (Δ), d-cis-diltiazem (O) or l-cis-diltiazem (□) were added to the incubation medium after maximum tension development in response to 35 mM KCl. The data represent the tissue response in percent of initial, maximum active tension. Decay of the maximum active tension was observed in simultaneously paired control tissue rings exposed to carrier solution alone. Appropriate corrections were made. Each point represents 4-12 coronary rings (±S.E.M.). In separate experiments, single doses of nitrendipine produced the same response at equilibrium as cumulative doses.

driven dog trabecular strips incubated at 30°C, nitrendipine caused a negative inotropic effect at concentrations above 10 nM (ED_{50}=1000 nM, data not shown) which is orders of magnitude higher than the dissociation constant obtained from radioligand binding (0.11 nM). The potency of nitrendipine in heart is consistent with that reported for nifedipine (9-11). These results suggest that the "receptor sites" for dihydropyridines are different in coronary artery and heart and/or that the "receptor-effector links" in the two tissues are different.

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