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COMPARISON OF HIGH AFFINITY BINDING OF CALCIUM CHANNEL BLOCKING DRUGS TO VASCULAR SMOOTH MUSCLE AND CARDIAC SARCOLEMMAL MEMBRANES *

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Abstract—The binding of the 1,4-dihydropyridine calcium channel blocker [^{3}H]nitrendipine to canine cardiac sarcolemmal and bovine aortic membranes was found to be rapid, specific, saturable, and reversible. Dissociation constants (K_d) determined by Scatchard analysis were 0.14 and 0.16 nM and the maximal numbers of binding sites (B_{max}) were 0.96 ± 0.2 and 0.08 ± 0.01 pmole/mg protein for cardiac and aortic membranes respectively. Values of K_d calculated from kinetic data were approximately 0.10 nM for both membrane preparations. Competition assays with the enantiomers of a nisoldipine derivative indicated that [^{3}H]nitrendipine binds stereoselectively. The order of potency of several nifedipine analogs for inhibition of binding of [^{3}H]nitrendipine to cardiac and aortic membranes paralleled their relative potencies for inhibition of contraction in smooth muscle. It is concluded that the high affinity binding sites for nitrendipine in bovine aortic smooth muscle membranes are similar to those of canine ventricular sarcolemma.

High affinity binding (K_d 0.1 to 2 nM) of [^{3}H] nitrendipine and [^{3}H]nimodipine has been found in membranes from cardiac [1–6], smooth [1, 2, 6–10] and skeletal muscles [11–13] as well as from brain [14–19]. However, the actions of these agents *in vivo* exhibit a high degree of tissue selectivity not reflected in the observed binding data [20, 21]. For example, the absolute potencies of nitrendipine for binding and inhibition of contraction are nearly identical in intestinal smooth muscle [1, 8], but the concentration of nitrendipine required to inhibit cardiac muscle contraction is usually 100–1000 times greater than the observed K_d in ligand binding studies [1–6, 9, 10].

One of the most imporant criterion for establishing that the binding of a given ligand occurs at a pharmacologically relevant site is the existence of the correct potency series for the binding of a series of active compounds that vary over a wide potency range [22]. Although vascular smooth muscle is presently the major therapeutic target for the 1,4-dihydropyridine Ca^{2+} antagonists [20, 21], this criterion has not been established for this tissue in ligand binding assays, nor has it been established for purified cardiac sarcolemmal membranes. In this report we present a comparison of the binding characteristics of [^{3}H]nitrendipine to partially purified sarcolemmal membranes from vascular smooth muscle and a highly purified sarcolemmal preparation from canine ventricle.

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MATERIALS AND METHODS

Membrane preparation. Fresh bovine aortae were obtained from a local abattoir, and the intima and adventia were carefully dissected away from the muscularis. The latter was cut into small cubes and homogenized in 5 vol. of 50 mM Tris–HCl, pH 7.4, at 4° using a Polytron homogenizer at a speed setting of 7. The homogenate was centrifuged at 1000 *g* for 10 min, and the supernatant fraction, after filtration through eight layers of cheesecloth, was centrifuged at 10,000 *g* for 10 min. The resultant supernatant fraction was centrifuged at 100,000 *g* for 60 min, and the pellet was resuspended in buffer, layered onto a sucrose gradient consisting of 15, 20, 40, and 60% sucrose (w/w), and centrifuged at 100,000 *g* for 2 hr. The band of vesicles which was stopped by the 20% sucrose layer was removed and used for binding assays.

Sarcolemmal membranes from canine ventricle were prepared as described by Jones *et al.* [23].

Protein concentrations were determined as described by Lowry *et al.* [24] using bovine serum albumin as standard.

Ligand binding. Equilibrium binding assays were carried out at 25° in 5 ml volumes containing 50 mM Tris–HCl (pH 7.4), and 20 μg/ml membrane protein for aorta or 2 μg/ml for cardiac sarcolemma. [^{3}H]-Nitrendipine (85 Ci/nmole, New England Nuclear Corp., Boston, MA) was added at the concentrations indicated in the figures. For Scatchard plot analysis [^{3}H]nitrendipine concentrations ranged from 0.02 to 0.56 nM in the presence and absence of non-labeled nitrendipine.

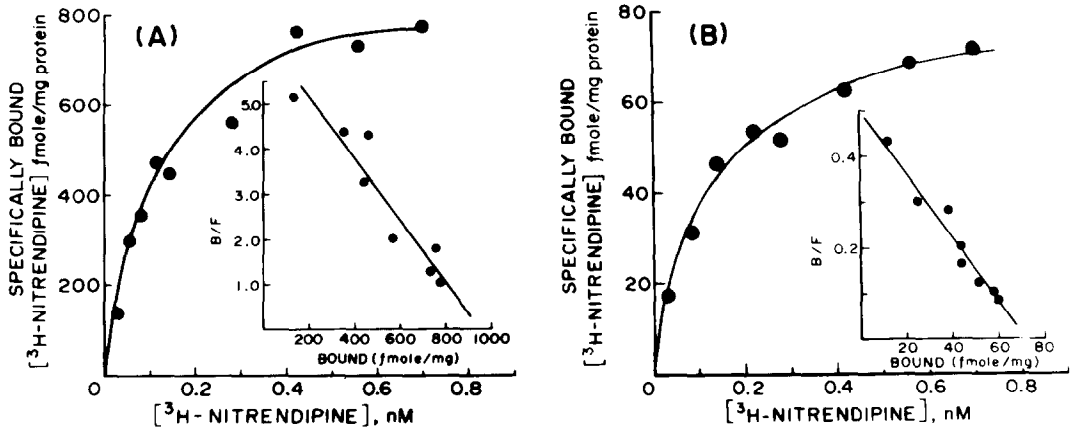

Fig. 1. Specific binding of [^{3}H]nitrendipine to membranes from canine cardiac sarcolemma (A) and bovine aorta (B) as a function of increasing concentrations of radioligand. Inset: Scatchard analysis of the means of pooled data. The mean ± S.E. for the dissociation constant (K_d) was 0.14 ± 0.02 nM for cardiac and 0.16 ± 0.02 nM for aorta. The maximal number of binding sites (B_{max}) was 0.96 ± 0.2 pmole/mg for cardiac and 0.08 + 0.01 pmole/mg for aorta.

Samples were incubated for 90 min and rapidly filtered by vacuum through Whatman GF/B filters; the filters were washed twice with 2.5 ml Tris buffer. The filters were placed in vials with 4 ml Biofluor and counted in a Searle liquid scintillation counter. The nifedipine analogs with the following substitution in the benzene moiety: H (unsubstituted), 3-methyl, 4-chloro, and 4-nitro, were synthesized in our laboratory (SUNY-Buffalo). Specific binding was defined as that displayed by 100 nM non-labeled nitrendipine. All procedures involving 1,4-dihydropyridines were carried out in the dark or under sodium vapor lamps. The isomers of 2,6-dimethyl-3-isopropyl-5-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (Bay e 6927) were obtained from Dr. H. Meyer of Bayer AG.

RESULTS

The binding of [^{3}H]nitrendipine to partially purified membranes derived from cardiac sarcolemma (Fig. 1A) and aorta smooth muscle (Fig. 1B) was specific and saturable over a concentration range of 0.02 to 0.7 nM. At 0.4 nM, approximately 50% of the total cpm represented specific binding for both membrane types. From Scatchard analyses of these saturation curves (insets to panels A and B), dissociation constants (K_d) of 0.14 ± 0.04 nM (mean ± S.E.M., N = 7) for heart and 0.16 ± 0.04 nM (mean ± S.E.M., N = 7) for aorta were calculated. The maximum numbers of binding sites (B_{max}) were 0.96 and 0.08 pmoles/mg protein for heart and aorta respectively. The Scatchard analysis in each case was linear, indicating that a single population of high affinity binding sites was present in both preparations. Hill analyses of these data gave linear plots with slopes of 1.03 for heart and 0.99 for aorta (not shown).

Kinetic analysis of [^{3}H]nitrendipine binding was carried out with each of these membrane preparations. The rate constants for association (k_1) and dissociation (k_{-1}) were calculated assuming that the

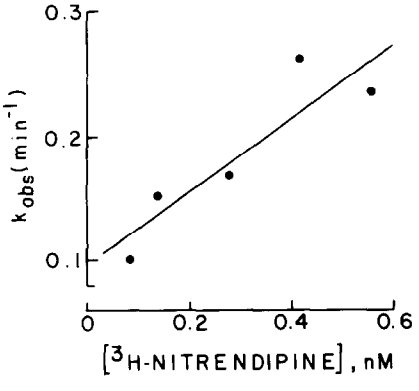

Fig. 2. Dependence of k_{obs} on [^{3}H]nitrendipine concentration for cardiac sarcolemmal membranes. The k_{obs} was determined from the slope of the pseudo first-order plot ln $[LR_e/LR_e - LR]$ versus time, with LR_e the concentration of [^{3}H]nitrendipine specifically bound at equilibrium and LR the concentration at time t. The slope of the line is equivalent to the k_1, 0.29 min^{-1} nM^{-1}.

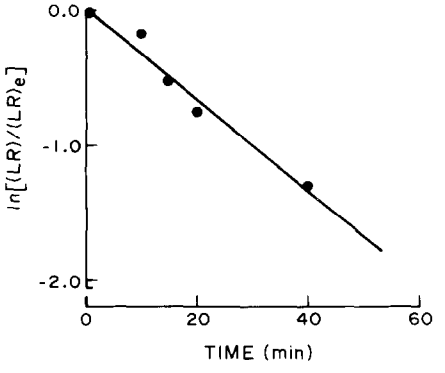

Fig. 3. Dissociation of [^{3}H]nitrendipine specifically bound to the cardiac sarcolemmal membranes. Membranes were equilibrated with [^{3}H]nitrendipine for 90 min and the dissociation was initiated by addition of excess nitrendipine (1 μM). Similar results were obtained by a dilution method. The k_{-1} calculated from the slope of the line was 0.035 min^{-1}.

Table 1. Summary of constants for nitrendipine binding to membranes from aortic smooth muscle and cardiac sarcolemma

Constant	Method	Aorta membrane	Cardiac sarcolemma
K_d (nM)	Scatchard (by saturation)	0.16 ± 0.02	0.14 ± 0.02
K_d (nM)	Scatchard (by competition)	0.11	0.29
k_{-1} (min^{-1})	Displacement (by saturation)	0.018	0.035
k_{+1} (min^{-1} nM^{-1})	Association	0.184	0.344
K_d (calculated)	k_{-1}/k_{-1}	0.10	0.10

binding reaction obeyed pseudo first-order kinetics [25]. The slope (k_1) of the plot k_{obs} versus the [^{3}H] nitrendipine concentration was 0.29 min^{-1} nM^{-1} for cardiac sarcolemma (Fig. 2). The dissociation of the drug from cardiac membranes was accomplished by displacement with non-labeled nitrendipine and gave a k_{-1} value of 0.035 min^{-1} (Fig. 3). Table 1 summarizes the binding constants for aortic and cardiac membranes and shows that these tissues bound nitrendipine with similar affinities. The values for K_d determined by competition assays were consistent with those calculated from the Scatchard plots and kinetic measurements.

The displacement of [^{3}H]nitrendipine by the non-labeled optical isomers Bay e 6927 in both heart and aorta membranes displayed stereoselectivity. The 4S(−) enantiomer of Bay e 6927 was approximately 300-fold more potent than the 4R(+) enantiomer in displacing [^{3}H]nitrendipine in cardiac sarcolemma. Similarly, aorta membrane nitrendipine binding sites discriminated between these isomers; the 4S(−) enantiomer had an IC_{50} value of 25 pM and the 4R(+) enantiomer, 15 nM (Fig. 4), also a 300-fold difference.

The structural specificity of [^{3}H]nitrendipine binding could be demonstrated in competition experiments with several analogs of nifedipine that exhibit pharmacological activity over a wide potency range. Specifically bound [^{3}H]nitrendipine was displaced by aortic and cardiac membranes by non-labeled nitrendipine and four nifedipine analogs with substitutions in the benzene moity as follows: unsubstituted, 3-methyl, 4-chloro and 4-nitro. Nitrendipine was more potent at displacing bound [^{3}H]-nitrendipine than any of the analogs, while the unsubstituted (data not shown) and 3-methyl derivatives showed similar potencies but one to two orders of magnitude less than nitrendipine. The 4-chloro and 4-nitro derivatives were the least effective at displacing [^{3}H]nitrendipine specifically bound to the membranes (data not shown).

The IC_{50} values for inhibition of [^{3}H]nitrendipine binding to aorta membranes by the optical isomers of Bay e 6927, and by nifedipine and its analogs, are compared to published IC_{50} values [8] for inhibition of K^+-induced contraction of smooth muscle in Fig. 5. The best fit line determined by linear regression analysis gave a satisfactory correlation coefficient ($r = 0.93$). Exactly the same results were obtained when cardiac sarcolemmal membranes were analyzed in this manner (data not shown).

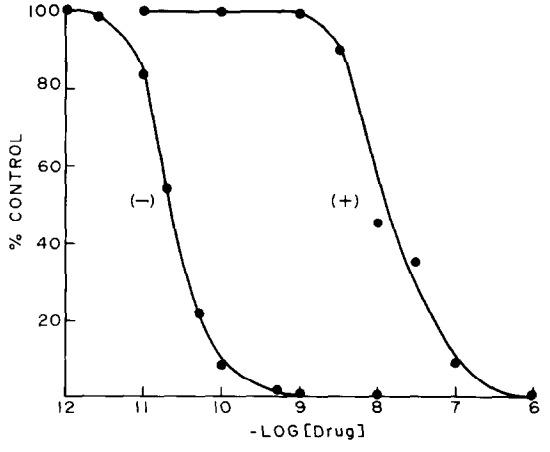

Fig. 4. Displacement of specifically bound [^{3}H]nitrendipine from aorta membranes by the (+) and (−) optical isomers of 2,6- dimethyl-3- isopropyl-5- methyl -4- (3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

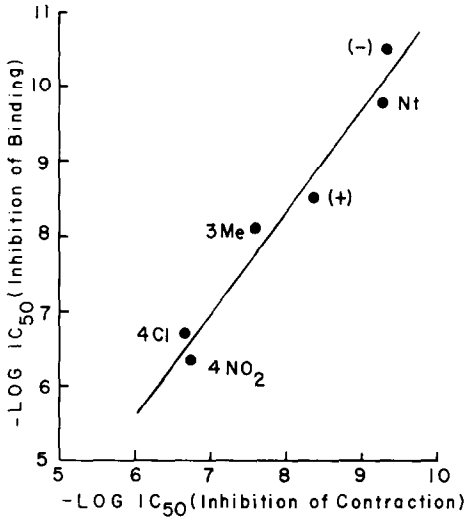

Fig. 5. Correlation of binding and pharmacological data. The IC_{50} values for various ligands in competition with [^{3}H] nitrendipine for binding to membranes from bovine aorta are plotted against the IC_{50} values for inhibition of tonic response of the ileal longitudinal muscle to KCl [8]. The best fit line, determined by linear regression analysis, has an $r = 0.93$. Compounds are 2,6-dimethyl-3,5-dicarbomethoxy-4-substituted phenyl-1,4-dihydropyridines with the following substituents: 4-chloro, 4-nitro, and 3-methyl; Nt, nitrendipine; (−), the 4S(−) enantiomer, and (+), the 4R(+) enantiomer, of Bay e 6927.

DISCUSSION

The present report describes a comparison of the binding characteristics of [^{3}H]nitrendipine to canine cardiac sarcolemmal membranes and bovine aorta smooth muscle membranes. The dissociation constants for these two membrane preparations, determined by both kinetic and equilibrium methods, were nearly identical (Table 1) and are similar to those reported by others for heart [5], brain [14] and ileum [1, 8]. Previous reports of [^{3}H]nitrendipine binding to membranes from coronary vessels [10] and aorta [6, 7], however, indicated a lower binding affinity that is of the same order of magnitude as that for skeletal muscle [11]. Differences in protein concentration and temperature may explain these discrepancies [26, 27].

Specific [^{3}H]nitrendipine binding displayed stereoselectivity for the optical isomers of a nitrendipine analog in both of these tissues (Fig. 4), and similar potencies for inhibition of binding of a series of nifedipine analogs were observed. These meet important criteria for the identification of a specific drug–receptor interaction. The good correlation between the inhibition of binding and the inhibition of contraction of guinea pig ileal smooth muscle (Fig. 5) provides further support for the view that the observed binding is pharmacologically relevant.

The major therapeutic site of action of the 1,4-dihydropyridines is the vasculature, cardiac tissue being less sensitive to these agents [20, 21, 28]. Other Ca^{2+} entry blocking drugs, such as verapamil and diltiazem, exhibit much less vascular selectivity. For this reason, it might be expected that the high affinity 1,4-diyhdropyridine binding sites in the myocardium would have a lower affinity than those in vascular smooth muscle. Currently available preparations of cardiac sarcolemma have been shown to be enriched in these binding sites, whereas cardiac mitochondria and a skeletal sarcoplasmic reticulum membrane fraction consisting largely of longitudinal tubules contain few if any of these high affinity sites [3]. A recent report by Williams and Jones [26] indicates that a ryanodine-sensitive cardiac sarcoplasmic reticulum subfraction bound nearly twice as much [^{3}H] nitrendipine as the sarcolemma. However, a denser subfraction of cardiac sarcoplasmic reticulum vesicles, which contained nearly twice as much Ca^{2+}-ATPase activity and phospholamban as the less dense subfraction, had the least [^{3}H]nitrendipine binding of all the subfractions. Skeletal muscle sarcoplasmic reticulum has been reported to contain lower affinity nitrendipine binding sites (K_d = 1.5 nM) [11] at a concentration approximately ten times that of the higher affinity sites that we have observed in the cardiac sarcolemma. Preliminary results from our laboratory confirm this finding. In our initial analysis we did not observe nitrendipine binding sites in skeletal sarcoplasmic reticulum having an affinity comparable to that of cardiac sarcolemma. Fosset *et al.* [12] recently reported that purified transverse tubules from skeletal muscle have a maximum of 56 pmoles of nitrendipine-binding sites per mg of protein, suggesting that the binding sites found in skeletal sarcoplasmic reticulum fractions are derived from the transverse tubules. The major difference between the cardiac sarcolemma and aortic membranes detected in the present study is in the binding site density, the aortic membranes having 10-fold fewer sites than the ventricular sarcolemma. Low B_{max} values have also been reported for membranes from coronary arteries [10] and aorta [7]. This apparently lower density of nitrendipine-binding sites in vascular smooth muscle than in the heart may be due partially to differences in the extent of purification of these membranes.

The similarity of the nitrendipine-binding affinities of cardiac and vascular membranes observed in this study is in marked contrast to the different potencies of this drug on calcium channels in these two tissues. The reason for this discrepancy is not clear. It is possible that the high affinity binding site in heart membranes may not be involved in the inhibition of slow inward current in cardiac tissue *in vivo*. Alternatively, the dihydropyridine-binding site may have been altered in some manner during preparation of cardiac membranes, resulting in a higher affinity conformation for binding to these drugs. Other possible explanations for this discrepancy have been discussed elsewhere [3, 9, 22, 28, 29]. The most likely explanation for the apparent discrepancy between binding and pharmacology is that the high affinity binding site represents binding to inactivated Ca^{2+} channels generated in isolated membranes (for review, see Janis and Triggle [21]).

The value of approximately 1 pmole nitrendipine-binding per mg protein for cardiac sarcolemma obtained in this study can be used to estimate the density of these sites on the surface of these membranes. This calculation, based on measurements of the lipid/protein ratio of these preparations and the average cross-sectional area of a phospholipid molecule as described elsewhere [30], gives an estimate of 0.5 to 5 nitrendipine-binding sites per μm^2 of lipid surface area. This value is similar to estimates of the density of calcium channels based on electrophysiological measurements for cardiac [31, 32] and neuronal cells [33].

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