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LOW AFFINITY BINDING SITES FOR 1,4-DIHYDROPYRIDINES IN MITOCHONDRIA AND IN GUINEA PIG VENTRICULAR MEMBRANES*

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Abstract—In this paper, we describe the occurrence of both high and low affinity sites for dihydropyridines in crude membrane preparations from guinea pig ventricular tissue. The physiological significance of the low affinity site (apparent dissociation constant = 76 ± 9 nM) is not currently known; it has, however, a binding capacity which was 300–1000 times that of the high affinity site and was resistant to heat denaturation. The magnitude of the binding to the low affinity site was affected by both the ionic strength of the medium and by the presence of divalent ions. Both unlabeled nitrendipine and nimodipine inhibited [^{3}H]nitrendipine binding at both sites, but verapamil and diltiazem only affected binding at the high affinity site. We also characterized, both kinetically and by equilibrium binding, a low affinity, heat-stable nitrendipine binding site in purified mitochondria. The B_{max} for this site was also dependent on ionic strength. This suggests the possibility that the low affinity site in crude membranes is due to mitochondrial contaminants and hence not directly related to voltage-dependent calcium channels.

A group of organic compounds collectively known as calcium channel antagonists block voltage-dependent calcium channels in a variety of cell types. Dihydropyridines, such as nitrendipine, have become widely used tools to study calcium channels both functionally and biochemically. When ventricular cells are depolarized from negative holding potentials, nitrendipine appears primarily to block calcium currents. However, at very negative membrane potentials, its action appears to be that of an activator of calcium currents [1, 2]. Mixed inhibitory and excitatory effects are seen at intermediate membrane potentials such that, at low concentrations of the nitrendipine, the first observable effect is an increase in calcium currents followed by a blockage of these currents [1]. Other dihydropyridines, such as Bay k 8644‡, enhance Ca^{2+} currents [1–4]. Comparison of the binding of radiolabeled nitrendipine with the inhibition of calcium currents in intact heart cells indicates that the dissociation constant for nitrendipine binding is 100- to 1000-fold smaller than the IC_{50} [5]. Part of this discrepancy can be explained by the voltage-dependence of the block by nitrendipine which suggests a preferential binding of nitrendipine to inactivated calcium channels [5–8]. Additional complications arise from the fact that most of the commonly used dihydropyridines are racemic mixtures. It has been shown recently that the enantiomers of the dihydropyridines 202-791 [9, 10] and Bay k 8644 [11] have opposing actions on calcium currents in intact cells. The discrepancy between radioligand binding and the IC_{50} for some of the optically pure dihydropyridine enantiomers, however, cannot be completely accounted for by voltage-dependent binding [12, 13]. Schwartz *et al.* [14] and Rogart *et al.* [15] have proposed the possibility that a low affinity [^{3}H]nitrendipine binding site may correspond to the inhibitory site of action of dihydropyridine on calcium channels.

In this paper we examine the binding of [^{3}H]nitrendipine to crude guinea pig ventricular membranes and present evidence for at least two binding sites. We also demonstrate the presence of a low affinity nitrendipine binding site in purified mitochondria. We suggest that the low affinity site in crude ventricular membranes arises from mitochondrial membranes and is, therefore, structurally unrelated to the voltage-dependent calcium channel.

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‡ Abbreviations: Bay k 8644, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate; B_{max}, maximum binding capacity in units of pmol per mg protein; IC_{50}, concentration of a compound which gives a half-maximal inhibition of the response; K_D, dissociation constant; K_I, apparent dissociation constant of an inhibitor; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PPO, 2,5-diphenyloxazole; and POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.

MATERIALS AND METHODS

Materials. [^{3}H]Nitrendipine (78 Ci/mmol) was purchased from New England Nuclear. [^{3}H]CGP-12177 (50.2 Ci/mmol) was purchased from Amersham. Unlabeled nitrendipine, Bay k 8644, nimodipine and purified nimodipine enantiomers were supplied by Miles Laboratory. [^{3}H]Dihydropyridine isothiocyanate (79 Ci/mmol) and [^{3}H]quinuclidinyl benzilate (39 Ci/mmol) were purchased from New England Nuclear.

Membrane preparation. Membranes were routinely prepared from guinea pig ventricular tissue. Ventricular tissue was removed from ten albino guinea pigs and placed in 20 ml of ice-cold Buffer I [25 mM KCl, 40 mM sodium tetraborate·HCl (pH 6.8), 5 mM EDTA] for 1 hr. The tissue was removed and minced with scissors. Buffer II [10 mM MOPS (pH 7.4), 10% sucrose, 1 mM aminobenzamidine, 1 μg/ml pepstatin, 0.5 mg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mM PMSF] was added to give 10 ml of buffer per g of tissue, and the tissue was homogenized three times for 15 sec each at high speed in a commercial Waring blender. The homogenate was centrifuged for 10 min at 4500 *g* in a JA 20 rotor in a Beckman J2-21 centrifuge. The supernatant fraction (S1) was removed and centrifuged at 30,000 rpm for 20 min in a 60 Ti rotor in an ultracentrifuge to give a pellet (P2) and a supernatant fraction (S2). The pellet from the second centrifugation (P2) was resuspended in 2 ml of 10 mM MOPS, 10% sucrose, containing protease inhibitors at the concentrations described above. Membranes (P2) were further purified by centrifugation on a 20–50% (w/w) sucrose gradient containing 10 mM MOPS (pH 7.4) and protease inhibitors for 15 hr at 25,000 rpm in an SW28 rotor, and 1-ml fractions were collected. Three peaks were isolated which contained high affinity [^{3}H]-nitrendipine binding sites. The lightest fraction banded at about 25% sucrose (A), the second peak banded at about 40% sucrose (B), and the third banded at 50% sucrose (C). The fraction used in each experiment is indicated in the figure legends.

Purification of mitochondria. Purified cardiac mitochondria were supplied by Dr. Louis Sordahl and were isolated from guinea pig ventricular tissue by the combination Polytron and Nagarse method of Sordahl and coworkers [16, 17].

[^{3}H]*Nitrendipine binding.* Membrane protein (40–75 μg) was incubated with [^{3}H]nitrendipine (0.025 to 1.00 nM) in 2.0 ml of 50 mM MOPS (pH 7.4), or 50 mM Tris–HCl (pH 7.4), at room temperature (26°) for 2 hr. Non-specific binding was determined in the presence of 1 μM unlabeled nitrendipine. Binding was terminated by rapid filtration on Whatman GF/B or GF/F filters with five washes of 5 ml of ice-cold distilled water. Filters were counted wet in 10 ml Beckman HP/b scintillant. Low affinity binding site assays using higher [^{3}H]nitrendipine concentrations (10–500 nM) were performed in 500 mM KCl, 50 mM MOPS (pH 7.4). Incubations were 2–4 hr at room temperature. To obtain these higher concentrations, the [^{3}H]nitrendipine was diluted either 1:50 or 1:100 with unlabeled nitrendipine, and non-specific binding was determined using 10 μM unlabeled nitrendipine.

[^{3}H]*CGP-12177 binding.* Membrane protein (25–100 μg) was incubated with the β-adrenergic antagonist, 4-(3-*t*-butylamino-2-hydroxypropoxyl)-[5,7-^{3}H]benzimidazol-2-one ([^{3}H]CGP-12177) (0.25 to 2.5 nM) in 2 ml of 50 mM MOPS (pH 7.4) at 25° for 2 hr. Non-specific binding was determined using 1 μM propranolol. Filtration was performed as described for [^{3}H]nitrendipine binding.

[^{3}H]*Quinuclidinyl benzilate (QNB) binding.* Membrane protein (20–60 μg) was incubated with [^{3}H]QNB in 5 ml of 5 mM $MgCl_2$, 50 mM MOPS (pH 7.4) at 25° for 2 hr. Non-specific binding was determined using 4 μM atropine. Filtration was performed on GF/F filters with three washes of 5 ml of ice-cold distilled water.

Enzyme assays. Succinate dehydrogenase activity was assayed by the method of King [18] as modified by Hochstadt *et al.* [19].

Data analysis. The binding data were fit to a one- or two-site model based on a non-linear least squares Marquardt method to estimate the free parameters, B_{max} and K_D. Parameter uncertainties were provided by χ^2 values. Scatchard analysis was performed using a least squares fit linear regression program.

RESULTS

Binding of [^{3}H]*nitrendipine to guinea pig ventricular membranes.* At low ionic strength, the binding of [^{3}H]nitrendipine to guinea pig ventricular membranes (Fig. 1) appears to be characterized by a single class of binding sites with an apparent dissociation constant of 0.12 ± 0.02 nM (N = 8). These

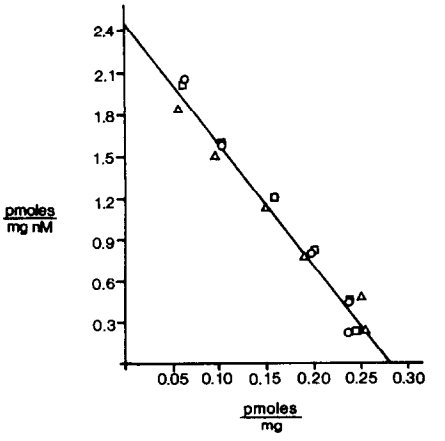

Fig. 1. High affinity [^{3}H]nitrendipine binding in a membrane preparation isolated from guinea pig ventricle. Scatchard analysis of equilibrium binding: Aliquots of membrane fraction C were incubated with [^{3}H]nitrendipine (0.3 to 1 nM) for 1 hr (○—○), 2 hr (□—□) and 3 hr (△—△) at room temperature in 2 ml of 50 mM Tris·HCl (pH 7.4). The final protein concentration was 0.05 mg/ml. The reactions were filtered and washed, and the radioactivity associated with the filter was determined as described in Materials and Methods. Non-specific binding was defined as the binding in the presence of 1 μM unlabeled nitrendipine for each concentration of [^{3}H]nitrendipine. K_D = 0.12 mM; B_{max} = 0.285 pmol/mg.

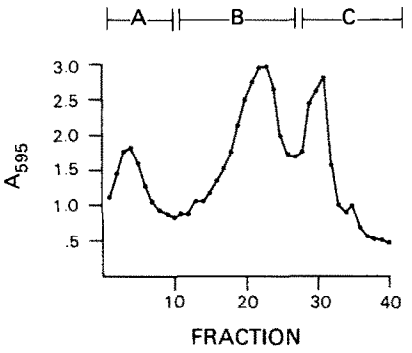

Fig. 2. Fractionation of guinea pig ventricular membranes on a 20–50% (w/w) continuous sucrose gradient. Separation on the gradient was performed as described in Materials and Methods. Aliquots (10 μl) of each 1-ml fraction were assayed for protein with 790 μl H_2O and 200 μl Biorad Protein Assay Concentrate. Brackets indicate fractions pooled for assays described in Table 1.

values are similar to those reported by a number of other laboratories (for review, see Ref. 20). The binding reached equilibrium by 1 hr and showed no further changes with time (Fig. 1). Crude membranes obtained by differential centrifugations as described in Methods were further fractionated on 20–50% (w/w) sucrose gradients and separated into three fractions (Fig. 2). High affinity nitrendipine binding sites were distributed fairly uniformly among the three fractions (Table 1). Similar findings have been reported by others [21–24]. Other than the number of binding sites per mg of protein, we have not detected any differences in the characteristics of the high affinity site in the different fractions. As seen in other laboratories [25–31], the binding of [^{3}H]nitrendipine to the high affinity site was enhanced by diltiazem and inhibited by verapamil (Fig. 3).

We have also examined the kinetics of [^{3}H]nitrendipine binding. Under pseudo first order conditions, the following rate equation applies:

$$\ln \frac{B_{eq}}{B_{eq} - B} = k_1 t \cdot [L_T][R_T]/B_{eq}$$

where B_{eq} is the concentration of [^{3}H]nitrendipine bound at equilibrium, B is the amount bound at time t, k_1 is the association rate constant, L_T is the concentration of [^{3}H]nitrendipine added, and R_T is the concentration of binding sites. The slope of a plot of $\ln B_{eq}/B_{eq} - B$ versus time is defined as k_{obs}. The association rate constant for binding to the high affinity site, determined from the slope of a plot of k_{obs} at different concentrations of [^{3}H]nitrendipine, was $6.1 \times 10^8\ M^{-1}\ min^{-1}$ (Fig. 4). The dissociation rate constant determined from the y intercept of this plot was $0.057\ min^{-1}$ which is in agreement with the dissociation rate constant of $0.044 \pm 0.009\ min^{-1}$ (N = 8) determined after the addition of excess unlabeled nitrendipine. Using the rate constants determined in Fig. 4, we calculated a value of 0.09 nM for the dissociation constant, in close agreement to the value obtained by equilibrium binding and similar to the values obtained in other laboratories [14, 32–36].

Low affinity nitrendipine binding sites have been reported by several laboratories [8, 13–15, 37–41]. We also found evidence for a low affinity binding site which was enriched in the denser membrane fractions (Table 1). These fractions were also

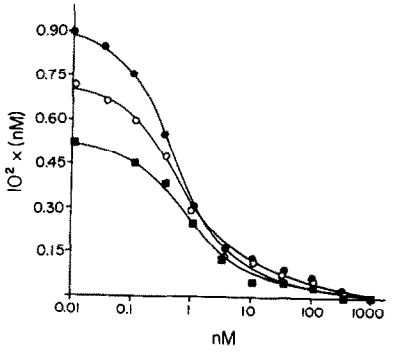

Fig. 3. Effects of diltiazem and verapamil on the inhibition of [^{3}H]nitrendipine binding by unlabeled nitrendipine at low ionic strength. Diltiazem (10 μM) or verapamil (100 nM) was added to aliquots of guinea pig ventricular membranes (P2) and incubated with 0.3 nM [^{3}H]nitrendipine and the indicated concentrations of unlabeled nitrendipine in 2 ml of 50 mM Tris–HCl (pH 7.4). The final protein concentration was 0.034 mg/ml. Non-specific binding was determined in the presence of 1 μM unlabeled nitrendipine. Each point represents the average of two determinations. Solid lines represent computer fits of the data. Control (○—○) $K_{D1} = 0.18$ nM, $B_{max1} = 9.84 \times 10^{-3}$ nM, $K_{D2} = 69.5$ nM, $B_{max2} = 0.143$ nM; control + 10 μM diltiazem (●—●) $K_{D1} = 0.18$ nM, $B_{max1} = 1.34 \times 10^{-2}$ nM, $K_{D2} = 69.5$ nM, $B_{max2} = 0.137$ nM; control + 100 nM verapamil (■—■) $K_{D1} = 0.18$ nM, $B_{max1} = 7.55 \times 10^{-3}$ nM, $K_{D2} = 69.5$ nM, $B_{max2} = 0.155$ nM.

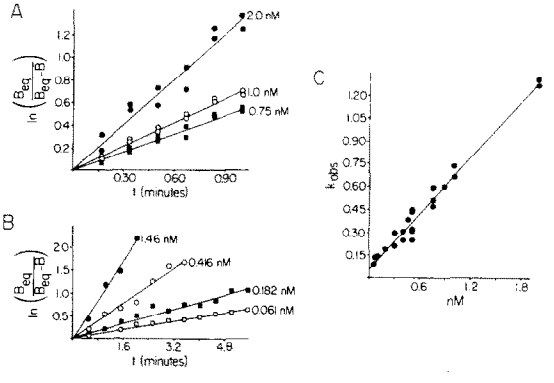

Fig. 4. Kinetics of [^{3}H]nitrendipine binding to membranes. (A) [^{3}H]Nitrendipine at the concentrations indicated was added to 56 μg/ml guinea pig membrane fraction C in 50 mM MOPS (pH 7.4), and 1-ml aliquots were filtered at 30-sec intervals. To determine the amount bound at equilibrium, duplicate 1-ml aliquots were filtered at 2 hr. (B) [^{3}H]Nitrendipine at the concentrations indicated was added to 125 μg/ml of guinea pig membrane fraction B in 50 mM MOPS (pH 7.4), and 500-μl aliquots were filtered at 10-sec intervals. To determine the amount bound at equilibrium, duplicate 500-μl samples were filtered at 2 hr. (C) k_{obs} was calculated from the slope of plots such as those shown in A and is plotted for a series of experiments using different [^{3}H]nitrendipine concentrations.

Table 1. Characterization of fractions from guinea pig ventricular membrane preparations

	High affinity nitrendipine B_{max} (pmol/mg)	Low affinity nitrendipine B_{max} (pmol/mg)	Succinate dehydrogenase activity (μmol/hr/mg)	[^{3}H]CGP-12177 B_{max} (pmol/mg) K_D = 0.3 nM	[^{3}H]QNB B_{max} (pmol/mg) K_D = 0.06 nM
Fraction A: 10–25% sucrose					
Preparation					
1	0.67	84	11	0.46	ND*
2	0.55	107	17	0.41	ND
3	0.24 ± 0.01	106 ± 13	8	0.27 ± 0.06	ND
4	0.35 ± 0.05	34 ± 17	5.5 ± 0.02	0.33 ± 0.03	3.25 ± 0.03
Fraction B: 25–40% sucrose					
Preparation					
1	0.33	254	29	0.19	ND
2	0.23	374	52	0.15	ND
3	0.19 ± 0.02	336 ± 13	37	0.03	ND
4	0.37 ± 0.03	180 ± 9	26.2 ± 0.4	0.07 ± 0.01	0.35 ± 0.02
Fraction C: 40–50% sucrose					
Preparation					
1	0.29	100	13	0.22	ND
2	0.25	209	35	0.13	ND
3	0.20 ± 0.03	215 ± 22	30	0.11 ± 0.03	ND
4	0.49 ± 0.07	74 ± 6	17.0 ± 0.5	0.06 ± 0.02	0.35 ± 0.07

B_{max} values were calculated from equilibrium binding at a single concentration of radioligand (in duplicate or triplicate) using the K_D values indicated (determined by Scatchard analysis). Where standard deviation values are given, N = 3.

* Not determined.

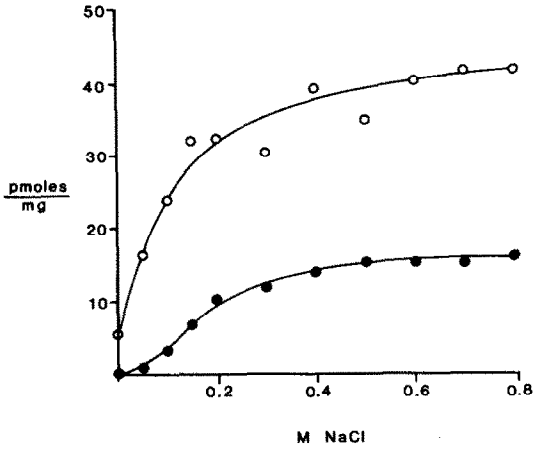

Fig. 5. [^{3}H]Nitrendipine binding to membranes and mitochondria as a function of salt concentration. Guinea pig ventricular membrane preparations (P2) and mitochondria were each incubated overnight at room temperature in 2 ml of 50 nM MOPS (pH 7.4) at the indicated salt concentration. [^{3}H]Nitrendipine (10 nM) was added and the mixture was incubated for 4 hr at room temperature. Final protein concentration in both cases was 0.02 mg/ml. Nonspecific binding was determined in the presence of 10 μM unlabeled nitrendipine. Each point represents the average of two determinations. Specific binding: mitochondria (O—O); P2 membranes (●—●).

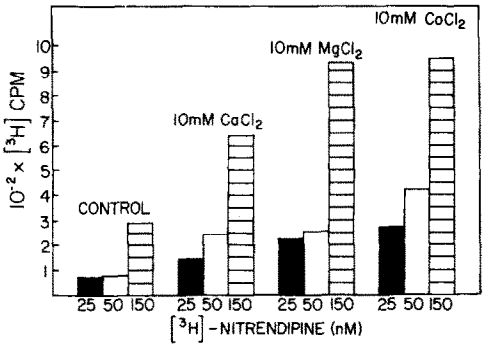

Fig. 6. Effect of divalent ions on [^{3}H]nitrendipine binding. Membranes (60 μg of P2 membrane protein) were incubated with [^{3}H]nitrendipine (25 nM, 50 nM, 150 nM) in 2 ml of 50 mM Tris (pH 7.4) containing 10 mM $CaCl_2$, 10 mM $CoCl_2$ or 10 mM $MgCl_2$ for 1 hr at room temperature. The ionic strength of each solution was made constant (200 mOs/kg) by the addition of NaCl.

enriched in succinate dihydrogenase activity and had relatively low amounts of surface membrane markers. [^{3}H]Nitrendipine binding to the low affinity site was most pronounced in buffers of high ionic strength (Fig. 5) or in those containing divalent cations (Fig. 6). Figure 5 shows the effect of increasing concentrations of NaCl on the binding of 10 nM [^{3}H]nitrendipine to crude membrane preparations and on binding to purified mitochondria (described below). Increasing the ionic strength caused an increase in the number of binding sites. The inhibition of [^{3}H]nitrendipine binding by increasing concentrations of unlabeled nitrendipine in high ionic strength buffer was characterized by at least two classes of binding sites. Low affinity binding, however, was not affected by either diltiazem or verapamil (data not shown). Dilution of [^{3}H]nitrendipine with unlabeled nitrendipine and the use of higher concentrations allowed the examination of the low affinity site by Scatchard analysis. The apparent K_D was 76 ± 9 nM (N = 3), and the B_{max} varied from 50 to 300 pmol per mg depending on the membrane preparation. When membranes were prepared under conditions which allowed osmotic lysis of mitochondria, there was a substantial enrichment in low affinity sites (Fig. 7).

In an attempt to determine the subcellular location of the low affinity nitrendipine binding site, we examined [^{3}H]nitrendipine binding to highly purified mitochondria. As shown in Fig. 8, mitochondria, when preincubated with 500 mM KCl and assayed at high ionic strength, had a low affinity nitrendipine binding

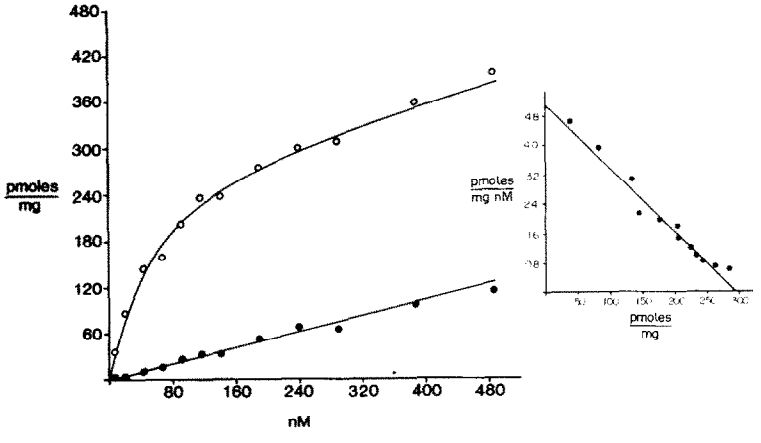

Fig. 7. Low affinity [^{3}H]nitrendipine binding to guinea pig membranes. Crude membrane fraction (20 μg/ml in triplicate) was incubated with [^{3}H]nitrendipine (10–500 nM at a specific activity of 0.78 Ci/mmol) in 50 mM MOPS (pH 7.4), 500 mM KCl at room temperature for 4 hr. Filtration was as previously described. Each point represents the average of three determinations. Total binding (O—O); nonspecific binding (●—●). Inset shows Scatchard analysis of specific binding. K_D = 57 nM; B_{max} = 295 pmol/mg.

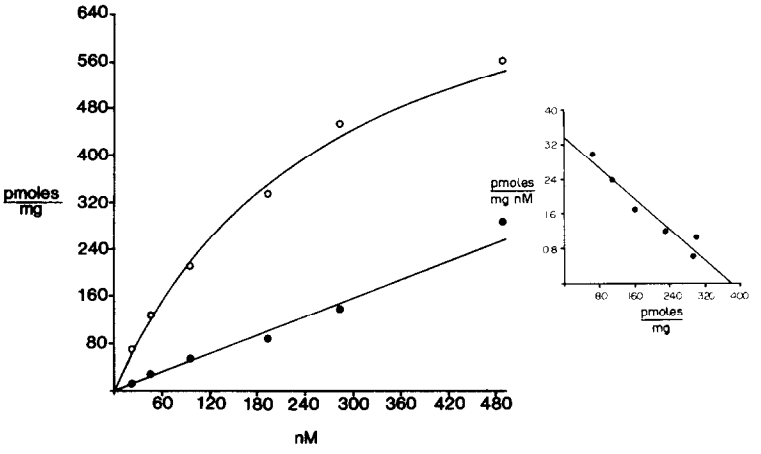

Fig. 8. [^{3}H]Nitrendipine binding to purified mitochondria. [^{3}H]Nitrendipine (10–500 nM) was added to 2 ml of 10 μg of purified mitochondria in 500 nM KCl, 50 mM MOPS (pH 7.4) and incubated for 4 hr at room temperature prior to filtration as previously described. Each point represents the average of two determinations. Total binding (○—○); non-specific binding (●—●). Inset shows Scatchard analysis of specific binding. K_D = 113 nM; B_{max} = 370 pmol/mg.

site. The magnitude of the [^{3}H]nitrendipine binding to this site was dependent on the ionic strength of the incubation buffer (Fig. 3). In 500 mM KCl, the apparent K_D for this site was 92 ± 13 nM and the B_{max} was 330 ± 55 pmol/mg (N = 4). [^{3}H]Nitrendipine binding to mitochondria was not affected by verapamil or diltiazem (Table 2). [^{3}H]Nitrendipine binding to both mitochondria and crude ventricular membranes as a function of the ionic strength of the total binding buffer is shown in Fig. 5.

To investigate the stereoselectivity of the mitochondrial binding site, inhibition of [^{3}H]nitrendipine binding was examined. In guinea pig cardiac membranes the relative potencies for these compounds to block high affinity [^{3}H]PN200-110 binding were (−)-nimodipine > (+)-nimodipine > (+)-Bay k 8644 > (−)-Bay k 8644 [12]. The K_I for inhibition of [^{3}H]nitrendipine binding to the low affinity site in mitochondria was found in two experiments to be 40 and 50 nM for (−)-nimodipine and 90 and 100 nM for (+)-nimodipine. At concentrations of 10 μM, (+)-Bay k 8644 gave only 10% inhibition, while (−)-Bay k 8644 produced no detectable inhibition.

Table 2. Effect of verapamil and diltiazem on low affinity [^{3}H]nitrendipine binding to guinea pig mitochondria

	% Control
10 μM Verapamil	98 ± 8
1 μM Verapamil	101 ± 8
0.1 μM Verapamil	113 ± 15
10 μM Diltiazem	102 ± 9
1 μM Diltiazem	116 ± 18
0.1 μM Diltiazem	103 ± 8

Purified guinea pig mitochondria (10 μg) were incubated for 2 hr at room temperature in 2 ml of buffer containing 50 mM MOPS (pH 7.4), 500 mM KCl, 10 nM [^{3}H]nitrendipine, and the indicated concentrations of verapamil or diltiazem. Specific [^{3}H]nitrendipine binding in the absence of inhibitor was 3.45 pmol/mg. Values are means ± SD, N = 3.

Neither the mitochondrial nor the low affinity membrane site was destroyed by heating. As shown in Fig. 9, there was an increase in the number of binding sites upon heat denaturation and a decrease in affinity. Kinetics of [^{3}H]nitrendipine binding to mitochondria is shown in Fig. 10. The association rate constant was found to be 4.6×10^5 M^{-1} min^{-1}, and the dissociation rate constant was 0.019 ± 0.003 min^{-1} (N = 9), giving rise to a calculated value for the dissociation constant of 41 nM in reasonable agreement with the value obtained from equilibrium binding.

DISCUSSION

In this paper we describe the binding properties of [^{3}H]nitrendipine in isolated guinea pig ventricular membranes. One of the major problems that has been encountered in the use of dihydropyridines in

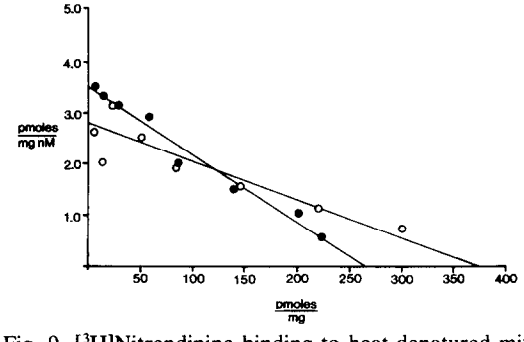

Fig. 9. [^{3}H]Nitrendipine binding to heat-denatured mitochondria. [^{3}H]Nitrendipine (10–500 nM) was incubated with either 20 μg of native mitochondria (●—●) or mitochondria heated to 80° for 15 min (○—○). The incubation, in 500 mM NaCl, 50 mM MOPS (pH 7.4), was for 3 hr at room temperature. Scatchard analysis of the data yields a K_D = 86 nM and a B_{max} = 265 pmol/mg for native mitochondria, and a K_D = 133 nM and a B_{max} = 375 pmol/mg for heat-denatured mitochondria.

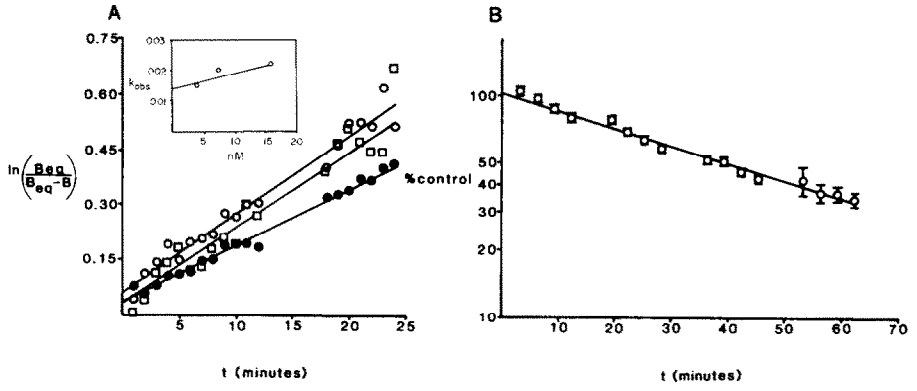

Fig. 10. Kinetics of [^{3}H]nitrendipine binding to mitochondria. (A) [^{3}H]Nitrendipine (500 nM) was added to 30 ml of purified mitochondria (10 μg protein/ml), and 500-μl aliquots were filtered through Whatman GF/F filters at the time intervals indicated. The filters were washed five times with 5 ml of ice-cold water. Equilibrium values were determined after 6 hr at room temperature. Inset: The observed rate constant, k_{obs}, is defined as $(k_1[L] + k_2)$ where k_1 is the association rate constant, k_2 is the dissociation constant, and $[L]$ is the concentration of radioligand. The k_{obs} plotted versus $[L]$ yields a straight line whose y intercept equals k_2 and whose slope equals k_1. (B) To initiate dissociation, unlabeled nitrendipine was added to 10 μM, and 500-μl aliquots were filtered at the time intervals indicated. Specific [^{3}H]nitrendipine bound at time = 0 was 1725 cpm/500-μl aliquot. Values are means ± SD.

the study of voltage-dependent calcium channels is the lack of correlation between the dihydropyridine concentration which produces half-maximal inhibition of voltage-dependent calcium currents and the apparent dissociation constants obtained from radioligand binding studies in tissues other than smooth muscle [5]. Part of the discrepancy can apparently be accounted for by the voltage dependence of the block by the dihydropyridine antagonists [6, 7]. The membrane potential in most binding experiments using isolated vesicles is different from that in the intact cells where the dose–response data are obtained. Other complications arise from the fact that the different enantiomers of the racemic mixture are acting functionally in opposite fashions [9–11] and that there is more than one dihydropyridine binding site in the membranes examined [13–15, 36–41].

We have examined the binding of [^{3}H]nitrendipine, [^{3}H]CGP-1277 and [^{3}H]QNB to fractionated cardiac ventricular membranes. We found an enrichment in binding of the β-adrenergic antagonist, [^{3}H]CGP-12177, and of the muscarinic cholinergic antagonist, [^{3}H]QNB, in membranes which band at a low density on sucrose gradients. Receptors for these ligands are located almost exclusively on the surface membranes [42–44]. The membranes banding at the low density, however, did not show the same fold enrichment for [^{3}H]nitrendipine high affinity binding sites as for the binding sites for the β-adrenergic and muscarinic ligands. This is in agreement with results reported by Williams and Jones [21], DePover *et al.* [22] and Rengasamy *et al.* [23] who found significant high affinity[^{3}H]nitrendipine binding in more dense fractions. The fraction containing the ryanodine-sensitive sarcoplasmic reticulum and the high density of [^{3}H]nitrendipine binding sites is possibly terminal cisternae [21]. Sarmiento *et al.* [44] reported no specific nitrendipine binding in a light cardiac sarcoplasmic reticulum fraction using a method designed to minimize contamination by sarcolemma and terminal cisternae. In skeletal muscle, nitrendipine binding is primarily located in t-tubules with very little binding in sarcoplasmic reticulum and surface membranes [45]. The high affinity nitrendipine binding sites in cardiac tissue could also be located in t-tubules or t-tubules adjacent to terminal cisternae. In support of this, Colvin *et al.* [46] in their studies on receptor site densities in cardiac sarcolemma suggest that there is a nonrandom distribution of nitrendipine binding sites on the sarcolemma and t-tubules.

Binding of [^{3}H]nitrendipine to cardiac membranes, when the assay was performed at low ionic strength, was apparently characterized by a single class of binding sites with a K_D of 0.12 nM. Binding to this site was enhanced by diltiazem and inhibited by verapamil. Similar results with verapamil and diltiazem have been reported by other laboratories [25–28]. From kinetic experiments, k_{obs} was found to be a linear function of the [^{3}H]nitrendipine concentration used, giving rise to a calculated association rate constant of $6.1 \times 10^8\ M^{-1}\ min^{-1}$ and a dissociation rate constant of $0.057\ min^{-1}$. The kinetics apparently reflect primarily the binding of the (−)-enantiomer, as Bellemann *et al.* [47] have reported a 20-fold difference in affinity of the two nitrendipine enantiomers. Biphasic association and dissociation such as described by Weiland and Oswald [48] using rat brain membranes were not observed in our experiments. These workers found monoexponential time courses at low concentrations of either (+)-[^{3}H]PN200-110 or [^{3}H]nitrendipine but at higher concentrations the association appeared biphasic. Although significant differences in the association kinetics may occur between brain membranes and cardiac membranes, the biphasic kinetics could reflect a contribution from a low affinity site described in this paper.

Nitrendipine binding to the high affinity site at

higher ionic strength was complicated by the presence of a low affinity site, a finding similar to that of Glossmann and Ferry [40]. The magnitude of nitrendipine binding to this site was dependent upon the ionic strength of the binding buffer and upon the presence of divalent cations. The ionic strength dependence of the low affinity binding site could be due to a salt-induced structural change in the molecule containing the binding site or to an unmasking of the site, perhaps by removal of peripheral proteins. These possibilities are currently being investigated. As the number of low affinity sites was 300–1000 times higher than that of the high affinity sites, the low affinity sites contributed to the total binding even at low nitrendipine concentrations. The physiological significance of the low affinity site is not known currently. Schwartz *et al.* [14] have speculated that it may represent the site of the negative inotropic action of nitrendipine, whereas the high affinity site represents the site of positive inotropic action. Striessnig *et al.* [49] have reported low affinity Ca^{2+} antagonist binding sites which are apparently linked to the nucleoside transporter in human erythrocytes. The low affinity binding site examined in this paper, although similar with respect to ionic strength dependence and heat stability, is apparently unrelated to the site studied by these workers in that it was not affected by *S*-(*p*-nitrobenzl)-6-thioinosine at concentrations as high as 10^{-5} M (conditions which inhibited the binding of dihydropyridines to the low affinity site studied by Striessnig *et al.* [49]). This compound inhibits binding to the erythrocyte nucleoside transporter [49]. We present evidence for the existence of an ionic strength dependent, low affinity binding site in purified mitochondria that had a K_D similar to that of the low affinity site in crude ventricular membranes and was not affected by diltiazem or verapamil. The enantiomers of nimodipine blocked [^{3}H]-(±)-nitrendipine binding to this site, but showed little stereoselectivity. The enantiomers of Bay k 8644 gave only slight inhibition of [^{3}H]nitrendipine binding to the mitochondrial site at the concentrations used. These results are similar to those observed by Vaghy *et al.* [41] for the inhibition of low affinity [^{3}H]nitrendipine binding to crude cardiac membranes. In addition, as also observed by Vaghy *et al.* [41], the site was not destroyed by heating, but rather appeared to have a higher binding capacity. Association and dissociation rate constants can be used to calculate a dissociation constant which is in close agreement with the value obtained from equilibrium binding. The presence of a low affinity site in crude ventricular membrane preparations, therefore, may be due to the presence of mitochondrial membrane contaminants. The possibility exists, however, that the binding of the dihydropyridines to mitochondria could give rise to secondary effects on Ca^{2+} channels, particularly if the binding caused release of Ca^{2+} or changes in internal pH or cyclic nucleotide levels. One possible candidate for the binding site for nitrendipine in mitochondria is the Na^+–Ca^{2+} carrier. Vaghy *et al.* [50] have shown that Ca^{2+} antagonists, such as diltiazem, verapamil, and nifedipine, selectively inhibit this protein in heart mitochondria. Another possibility is the adenine nucleotide translocase. In isolated mitochondria, creatine kinase is bound by electrostatic forces [50] to the outside of the inner mitochondrial membrane where it appears to interact with the adenine nucleotide translocase [51–56]. Elevated KCl concentrations remove the creatine kinase from the mitochondria [57, 58]. A similar mechanism may be exposing the low affinity dihydropyridine binding site in mitochondria as this site can only be detected at high ionic strength. This is currently being investigated.

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REFERENCES

1. A. M. Brown, D. L. Kunze and A. Yatani, *J. Physiol. Lond.* **357**, 59P (1984).
2. P. Hess, J. B. Lansman and R. W. Tsien, *Nature, Lond.* **311**, 538 (1984).
3. M. Schramm, G. Thomas, R. Towart and G. Frankowiak, *Nature, Lond.* **303**, 535 (1983).
4. M. Schramm, G. Thomas, R. Towart and G. Frankowiak, *Arzneimittel-Forsch.* **33**, 1268 (1983).
5. K. S. Lee and R. W. Tsien, *Nature, Lond.* **302**, 790 (1983).
6. M. C. Sanguinetti and R. S. Kass, *Circulation Res.* **55**, 336 (1984).
7. B. P. Bean, *Proc. natn. Acad. Sci. U.S.A.* **81**, 6388 (1984).
8. D. L. Kunze, M. J. Hawkes, S. L. Hamilton and A. M. Brown, *Biophys. J.* **47**, 264a (1985).
9. R. P. Hof, U. T. Rüegg, A. Hof and A. Vogel, *J. cardiovasc. Pharmac.* **7**, 689 (1985).
10. J. S. Williams, I. L. Grupp, G. Grupp, P. L. Vaghy, L. Dumont, A. Schwartz, A. Yatani, S. Hamilton and A. M. Brown, *Biochem. biophys. Res. Commun.* **131**, 13 (1985).
11. G. Frankowiak, M. Bechom, M. Schramm and G. Thomas, *Eur. J. Pharmac.* **114**, 223 (1985).
12. S. L. Hamilton, A. Yatani, K. Brush, A. Schwartz and A. M. Brown, *Molec. Pharmac.* **31**, 221 (1987).
13. D. L. Kunze, S. L. Hamilton, M. J. Hawkes and A. M. Brown, *Molec. Pharmac.* **31**, 401 (1987).
14. A. Schwartz, I. L. Grupp, G. Grupp, J. S. Williams and P. L. Vaghy, *Biochem. biophys. Res. Commun.* **125**, 387 (1984).
15. R. B. Rogart, A. de Bruyn Kops and V. J. Dzau, *Proc. natn. Acad. Sci. U.S.A.* **83**, 7452 (1986).
16. L. A. Sordahl and M. L. Stewart, *Circulation Res.* **47**, 814 (1980).
17. L. A. Sordahl, *Methods in Studying Cardiac Membranes* (Ed. N. S. Dhalla), Vol. 1, pp. 65–74. CRC Press, Boca Raton, FL (1984).
18. T. E. King, *Meth. Enzym.* **10**, 216 (1967).
19. J. Hochstadt, D. C. Quinlan, R. L. Rader, C-C. Li and D. Dowd, *Methods in Membrane Biology* (Ed. E. D. Korn), Vol. 5, pp. 117–62. Plenum Press, New York (1975).
20. D. J. Triggle and R. A. Janis, *Nitrendipine* (Eds. A. Scriabine, S. Vanov and K. Deck), pp. 33–52. Urban & Schwarzenberg, Baltimore, MD (1984).
21. L. T. Williams and L. R. Jones, *J. biol. Chem.* **258**, 5344 (1983).
22. A. DePover, S. W. Lee, M. A. Matlib, K. Whitmer, B. A. Davis, T. Powell and A. Schwartz, *Biochem. biophys. Res. Commun.* **113**, 185 (1983).
23. A. Rengasamy, J. Ptasienski and M. M. Hosey, *Biochem. biophys. Res. Commun.* **126**, 1 (1985).

24. D. D. Doyle, T. J. Kamp, H. C. Palfrey, R. J. Miller and E. Page, *J. biol. Chem.* **261**, 6556 (1986).
25. F. J. Ehlert, E. Itoga, W. R. Roeske and H. I. Yamamura, *Biochem. biophys. Res. Commun.* **104**, 937 (1982).
26. F. J. Ehlert, W. R. Roeske, E. Itoga and H. I. Yamamura, *Life Sci.* **30**, 2191 (1982).
27. A. DePover, M. A. Matlib, S. W. Lee, G. P. Dubé, I. L. Grupp, G. Grupp and A. Schwartz, *Biochem. biophys. Res. Commun.* **108**, 110 (1982).
28. K. M. M. Murphy, R. J. Gould, B. L. Largent and S. H. Snyder, *Proc. natn. Acad. Sci. U.S.A.* **80**, 860 (1983).
29. G. T. Bolger, P. Gengo, R. Klockowski, E. Luchowski, H. Siegel, R. A. Janis, A. M. Triggle and D. J. Triggle, *J. Pharmac. exp. Ther.* **225**, 291 (1983).
30. H. Glossmann, D. R. Ferry and C. B. Boschek, *Naunyn-Schmiedeberg's Archs Pharmac.* **323**, 1 (1983).
31. H. I. Yamamura, H. Schoemaker, R. G. Boles and W. R. Roeske, *Biochem. biophys. Res. Commun.* **108**, 640 (1982).
32. P. Bellemann, D. Ferry, F. Lübbecke and H. Glossmann, *Arzneimittel-Forsch.* **31**, 2064 (1981).
33. H. Glossmann, D. R. Ferry, F. Lübbecke, R. Mewes and F. Hofmann, *Trends pharmac. Sci.* **3**, 431 (1982).
34. R. J. Gould, K. M. M. Murphy and S. H. Synder, *Proc. natn. Acad. Sci. U.S.A.* **79**, 3656 (1982).
35. C. R. Triggle, D. K. Agrawal, G. T. Bolger, E. E. Daniel, C. Y. Kwan, E. M. Luchowski and D. J. Triggle, *Can. J. Physiol. Pharmac.* **60**, 1738 (1982).
36. W. P. Schilling, S. L. Hamilton, A. Yatani, K. Brush and A. M. Brown, *Biophys. J.* **47**, 264 (1985).
37. J. D. Marsh, E. Loh, D. Lachance, W. H. Barry and T. W. Smith, *Circulation Res.* **53**, 539 (1983).
38. M. J. Litzinger and D. E. Brenneman, *Biochem. biophys. Res. Commun.* **127**, 112 (1985).
39. E. Van Alstyne, R. M. Burch, R. G. Knickelbein, R. T. Hungerford, E. J. Gower, J. G. Webb, S. L. Poe and G. E. Lindenmayer, *Biochim. biophys. Acta* **602**, 131 (1980).
40. H. Glossmann and D. R. Ferry, *Drug Development and Evaluation* (Eds. A. Fleckenstein, K. Hashimoto, M. Hermann, A. Schwartz and L. Seipel), Vol. 9, pp. 63–98. Gustav Fisher, Stuttgard, (1983).
41. P. L. Vaghy, G. P. Dubé, I. L. Grupp, G. Grupp, J. S. Williams, Y. H. Baik and A. Schwartz, *Bayer-Symposium IX* (Eds. A. Fleckenstein, C. Van Breemann, R. Grob and F. Hoffmeister), pp. 156–84. Springer, Berlin (1985).
42. L. R. Jones, H. R. Besch, J. W. Fleming, M. M. McConnaughey and A. M. Watanabe, *J. biol. Chem.* **254**, 530 (1979).
43. L. R. Jones, S. W. Maddock and H. R. Besch, Jr., *J. biol. Chem.* **255**, 9971 (1980).
44. J. G. Sarmiento, R. A. Janis, R. A. Colvin, D. J. Triggle and A. M. Katz, *J. molec. cell. Cardiol.* **15**, 135 (1982).
45. M. Fosset, E. Jaimovich, E. Delpont and M. Lazdunski, *J. biol. Chem.* **258**, 6086 (1983).
46. R. A. Colvin, T. F. Ashavaid and L. G. Herbette, *Biochim. biophys. Acta* **812**, 601 (1985).
47. P. Bellemann, A. Schade and R. Towart, *Proc. natn. Acad. Sci. U.S.A.* **80**, 2356 (1983).
48. G. A. Weiland and R. E. Oswald, *J. biol. Chem.* **260**, 8456 (1985).
49. J. Striessnig, G. Zernig and H. Glossmann, *Eur. J. Biochem.* **150**, 67 (1985).
50. P. L. Vàghy, J. D. Johnson, M. A. Matlib, T. Wang and A. Schwartz, *J. biol. Chem.* **257**, 6000 (1982).
51. M. Müller, R. Moser, D. Cheneval and E. Carafoli, *J. biol. Chem.* **260**, 3839 (1985).
52. V. A. Saks, G. B. Chernousova, D. E. Gukovsky, V. N. Smirnov and E. I. Chazov, *Eur. J. Biochem.* **57**, 273 (1975).
53. W. E. Jacobus and V. A. Saks, *Archs Biochem. Biophys.* **219**, 167 (1982).
54. R. Moreadith and W. Jacobus, *J. biol. Chem.* **257**, 899 (1982).
55. V. A. Saks, A. V. Kuznetsov, V. V. Kupriyanov, M. V. Miceli and W. E. Jacobus, *J. biol. Chem.* **260**, 7757 (1985).
56. W. E. Jacobus, *A. Rev. Physiol.* **47**, 707 (1985).
57. W. C. Wenger, M. P. Murphy, G. P. Brierley and R. A. Altschuld, *J. Bioenerg. Biomemb.* **17**, 295 (1986).
58. V. A. Saks, Z. A. Khuchua, A. V. Kuznetsov, V. I. Veksler and V. G. Sharov, *Biochem. biophys. Res. Commun.* **139**, 1262 (1986).