Effects of Bay k 8644, a Dihydropyridine Analog, on \[^{3}\text{H}]\text{Nitrendipine} Binding to Canine Cardiac Sarcolemma and the Relationship to a Positive Inotropic Effect

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SUMMARY. Equilibrium dissociation constants of Bay k 8644, a calcium agonist, and nitrendipine, a calcium antagonist, were determined in canine cardiac sarcolemma. The equilibrium dissociation constant for Bay k 8644 was compared to the concentration that produced a fifty percent increase, and the equilibrium dissociation constant for nitrendipine was compared to the concentration that produced a fifty percent decrease, in contractile force in canine heart trabecular muscle. Both saturation and inhibition binding data suggest that Bay k 8644 and nitrendipine bind to and compete for a high affinity dihydropyridine-binding site present in isolated cardiac sarcolemma preparations. The equilibrium dissociation constant (7-10 nM) and concentration that produced a fifty percent increase in contractile force in the canine trabecular muscle (30 ± 8 nM) of Bay k 8644 were in a similar concentration range, but the equilibrium dissociation constant (0.29 ± 0.025 nM) of nitrendipine binding was more than a thousand-fold lower than the concentration that produced a fifty percent decrease in contractile force in canine trabecular muscle (613 ± 109 nM). These data suggest that binding of Bay k 8644 to high affinity binding sites is pharmacologically relevant, and is related to a positive inotropic effect. (Circ Res 55: 549-553, 1984)

Several dihydropyridine calcium antagonists have been shown to cause relaxation of vascular smooth muscle and negative inotropy in the heart (Fleckenstein, 1983; Janis and Triggle, 1983; Millard et al., 1983; Schwartz and Triggle, 1984). Among them, the most extensively studied compounds are nifedipine, nitrendipine, nimodipine, and nisoldipine. The primary site of action of calcium antagonists is attributed to a calcium channel, in that these drugs inhibit calcium influx into the cells (Katz, 1983; Reuter, 1983; Cauvin et al., 1983; Nayler and Horowitz, 1983; Janis and Scriabine, 1983; Schwartz and Triggle, 1984). Findings of these investigators are supported by results, obtained by a suction pipette voltage clamp technique, showing that nitrendipine inhibits calcium currents in single heart cells (Lee and Tsien, 1983).

Recently, a novel dihydropyridine derivative, Bay k 8644, was synthesized which has effects opposite to those of the calcium antagonists. This compound causes vasoconstriction and positive inotropy in the heart, apparently by competing for a nifedipine-binding site (Schramm et al., 1983a, 1983b). In contrast to the effect of nitrendipine, Bay k 8644 stimulates calcium currents in single heart cells (Hess et al., 1984; Sanguinetti and Kass, 1984; Cohen and Chung, 1984).

In several studies, labeled calcium antagonists have been used to demonstrate the presence of a high affinity dihydropyridine-binding site in cardiac membranes (Bellemann et al., 1981; Bolger et al., 1982; DePover et al., 1982; Janis et al., 1982; Murphy and Snyder, 1982; Sarmiento et al., 1983; DePover et al., 1983a, 1983b). It has recently been suggested that nimodipine and Bay k 8644 may bind to the same drug receptor site in skeletal muscle microsomes (Glossmann et al., 1983). To determine whether there is a relationship between binding and pharmacological potency, the binding of Bay k 8644 and nitrendipine to isolated canine cardiac sarcolemma was compared with their pharmacological effects on dog heart.

Methods

Isolation and Characterization of Cardiac Sarcolemma

Dog heart sarcolemma was isolated by the method described by Van Alstyne et al. (1980). The protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard. Ouabain-sensitive Na⁺,K⁺-ATPase activity was measured as described by Lee et al. (1983).

\[^{3}\text{H}\]Nitrendipine-Binding Assays

Dog heart sarcolemma (~25 μg protein) was incubated in a total volume of 1 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl and 1 mM CaCl₂ at 37°C.
with varying concentrations of [3H]nitrendipine (70 Ci/mmoll, New England Nuclear), both in the absence and in the presence of Bay k 8644. After 50 minutes of incubation, the samples were filtered through Whatman GF/C filters with the aid of a Brandell Cell Harvester (Biomedical Research Lab). The filters were washed three times with 5 ml of ice cold 50 mM Tris-HCl buffer, pH 7.4, and placed into scintillation vials; 8 ml of a scintillation "cocktail" (Research Products International Corp.) were added, and radioactivity was counted with a Beckman LS 8100 liquid scintillation counter at 39–42% efficiency. All experiments were carried out in triplicate. The [3H]nitrendipine binding in the presence of 10−7 M nonlabeled nitrendipine was defined as nonspecific binding and was subtracted from the total binding to obtain specific binding.

According to our experience, both nitrendipine and Bay k 8644 adhere to plastic and glass surfaces. Therefore, special care was taken to saturate pipet tips before dilutions of stock solutions were made. All experiments were performed under a sodium lamp to prevent deterioration of these light-sensitive dihydropyridine derivatives. Kp and Bmax, values were calculated from both Scatchard analysis (Scatchard, 1949) and, in separate experiments, a displacement assay (Akera and Cheng, 1977; Cheng and Prusoff, 1973) of specific binding data using linear regression analysis, and by the iterative, nonlinear least squares computer program of Munson and Rodbard (1980) converted to Applesoft by M. H. Teicher, (MED-58) ligand program (Biomedical Computing Technology Information Center, Nashville, Tennessee). Nonlabeled nitrendipine and Bay k 8644 were supplied by Miles Pharmaceuticals, courtesy of Dr. Alexander Scriabine.

Contractility Measurement

Right ventricular and atrial trabecular muscles of dog heart were suspended in Krebs-Henseleit solution containing 2 mM CaCl2 and 5.9 mM KCl, pH 7.4. Most of the experiments were carried out at 35°C, although some were also done at 30–33°C, with very little difference. The trabeculae were stimulated at 1 Hz and the contractile force was measured as described previously (Millard et al., 1982; Lee et al., 1983). Concentrations of Bay k 8644 and nitrendipine were added in a cumulative manner to the tissue for 25–30 minutes, during which time equilibrium was attained. We emphasize that isolated dog trabeculae are generally more difficult to handle than similar tissues from small animals. Hence, greater variability is to be expected and the data derived should be viewed as a "range of values" rather than exact numbers.

Results

The activity of ouabain-sensitive Na+,K+–ATPase was determined in order to characterize our sarcolemma preparations. The Na+,K+–ATPase activity of 129 ± 11 µmol Pi/mg per hour (n = 7) was consistent with a relatively purified canine sarcolemma preparation (Johnson et al., 1982; Lee et al., 1983).

In saturation binding experiments, the [3H]nitrendipine concentration was varied between 0.05 and 1.1 nM. Figure 1 demonstrates total and nonspecific binding of [3H]nitrendipine to cardiac sarcolemma under control conditions. The nonspecific binding was 15% of the total binding at a [3H]nitrendipine concentration of 1.1 nM.

Scatchard plots of specific binding data revealed straight lines both in the absence (r = −0.99) and in the presence (r = −0.98) of 5 nM Bay k 8644 (Fig. 2). In control experiments, a Kp of 0.32 nM and a Bmax of 1.33 pmol/mg protein were obtained for nitrendipine. The presence of 5 nM Bay k 8644 increased the Kp of nitrendipine to 0.6 nM, but it did not influence its Bmax (1.32 pmol/mg protein). Hill plots of specific binding data also resulted in straight lines with slopes of 0.99 in the absence and 0.97 in the presence of Bay k 8644 (Fig. 3). These data suggest an apparent competition between Bay k 8644 and nitrendipine for the same single class of high affinity dihydropyridine-binding sites. Saturation binding experiments carried out with varying concentrations of Bay k 8644 (5–10 nM) indicate that the effect of this calcium agonist on high affinity dihydropyridine binding is concentration dependent (data not shown). Analysis of saturation binding

![Figure 1](image)

**Figure 1.** Saturation binding of [3H]nitrendipine ([3H]NTD) to isolated canine cardiac sarcolemma. Total binding (○) and nonspecific binding (△) values represent the mean of triplicate measurements in a typical experiment.

![Figure 2](image)

**Figure 2.** Scatchard plots of specific [3H]nitrendipine binding to cardiac sarcolemma. The amount of bound [3H]nitrendipine ([3H]NTD) to 23 µg sarcolemmal protein is indicated on the horizontal axis and bound:free (B:F) ratio is indicated on the vertical axis. The demonstrated values represent the mean of triplicate measurements in a typical experiment. The upper curve (△) demonstrates [3H]nitrendipine binding in the absence of Bay k 8644. The lower curve (○) indicates [3H]nitrendipine binding in the presence of 5 nM Bay k 8644.
Nitrendipine has been used to demonstrate dihydropyridine-binding sites (Table 1). On the other hand, the concentration of Bay k 8644 was about 30 times less effective compared to nonlabeled nitrendipine (Fig. 4). Log-logit plots of inhibition binding data were linear and parallel to each other (inset, Fig. 4). The slope was 2.55 in the case of nonlabeled nitrendipine and Bay k 8644, respectively. The inhibition binding data analyzed by the nonlinear least squares method resulted in a $K_D$ of 0.39 ± 0.025 nM ($n = 4$) and a $B_{max}$ of 1.4 ± 0.1 pmol/mg protein ($n = 4$) for nitrendipine. The $K_i$ value for Bay k 8644 binding calculated from the saturation binding data was 7.44 ± 1.02 nM ($n = 4$).

Both nonlabeled nitrendipine and Bay k 8644 completely inhibited specific $[^3H]$nitrendipine binding; Bay k 8644 was about 30 times less effective than nonlabeled nitrendipine (Fig. 4). Log-logit plots of inhibition binding data were linear and parallel to each other (inset, Fig. 4). The slope was 2.55 in the case of nonlabeled nitrendipine, and 2.26 when Bay k 8644 was used for displacement. Hill plots of these data were also linear with $n_H$ of 1.11 and 0.92 for nonlabeled nitrendipine and Bay k 8644, respectively. The inhibition binding data analyzed by the nonlinear least squares method resulted in a $K_D$ of 0.31 ± 0.011 nM ($n = 4$) and a $B_{max}$ of 1.86 ± 0.16 pmol/mg protein ($n = 4$) for nitrendipine. By this procedure, the estimated $K_i$ value for Bay k 8644 binding was 10.1 ± 0.9 nM ($n = 3$). These binding constants are very similar to those obtained in saturation binding experiments.

The concentration of Bay k 8644 which caused a half maximal increase in contractile force (ED$_{50}$) was in a range similar to the apparent dissociation constant of this drug for the high affinity dihydropyridine-binding sites (Table 1). On the other hand, the nitrendipine concentration which caused a half maximal decrease of contractile force (I$_{50}$) was more than 1000 times higher than the apparent dissociation constant of nitrendipine for the high affinity dihydropyridine-binding sites (Table 1).

**Discussion**

Since the work of Bellemann et al. (1981), $[^3H]$nitrendipine has been used to demonstrate dihydropyridine binding to various membrane and tissue preparations. The $K_D$ of 0.3 nM obtained in this study for $[^3H]$nitrendipine binding to isolated canine cardiac sarcolemma is very similar to the $K_D$ values reported for various cardiac membrane preparations (DePover et al., 1982; Janis et al., 1982; Murphy and Snyder, 1982). In general, the $B_{max}$ values reported by these authors are somewhat lower than those we found in the present study. Our $B_{max}$ values are, however, very close to the one obtained recently in isolated canine cardiac sarcolemma preparations by Sar- miento et al. (1983). The differences in the published $B_{max}$ values for $[^3H]$nitrendipine binding to cardiac membrane fractions are probably due to different incubation conditions and/or a different degree of membrane purity.

Our saturation and inhibition binding data, taken together with the structural similarity of Bay k 8644 and nitrendipine, suggest that these two dihydropyridine derivatives compete for high affinity dihydropyridine-binding sites present in isolated cardiac sarcolemma preparations. Similar suggestions were made by Schramm et al. (1983a, 1983b) on the basis of pharmacological studies, and by Glossmann et al. (1983), who measured the effect of Bay k 8644 on $[^3H]$nitrendipine binding to guinea pig skeletal muscle microsomes.

Our radioligand-binding data may provide a molecular explanation for the pharmacological effects of these drugs. To correlate radioligand binding data with the pharmacological effects requires that the $K_D$ of the drug for the binding site (receptor) measured in vitro be similar to the pharmacologically effective concentrations in the same tissue (Titeler, 1983). The ED$_{50}$ values of Bay k 8644 we found for increasing the force of contraction of isolated dog heart preparations, 30.5 nM and 29.5 nM for atria and ventricle, respectively, are in a similar concentration range as the apparent $K_i$ for Bay k 8644 binding to isolated canine cardiac sarcolemma, 7–10 nM. Measuring whole cell calcium current, by a patch clamp procedure, the estimated $K_D$ value for Bay k 8644 binding was 4 nM ($n = 4$) and a $B_{max}$ of 1.86 ± 0.16 pmol/mg protein (n = 4) for nitrendipine. By this method resulted in a KD of 0.29 ± 0.025 nM (DePover et al., 1982; Janis et al., 1982; Murphy and Snyder, 1982). In general, the $B_{max}$ values reported by these authors are somewhat lower than those we found in the present study. Our $B_{max}$ is, however, very close to the one obtained recently in isolated canine cardiac sarcolemma preparations by Sar- miento et al. (1983). The differences in the published $B_{max}$ values for $[^3H]$nitrendipine binding to cardiac membrane fractions are probably due to different incubation conditions and/or a different degree of membrane purity.

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clamp technique, in adult guinea pig ventricular myocytes, A. M. Brown, D. L. Kunze, and A. Yatani (Nature, submitted) have reported an ED$_{50}$ of 33 nM (22–25°C) (A. M. Brown, personal communication). Hess et al. (1984) reported an ED$_{50}$ of 50–100 nM in similar experiments. These results suggest a correlation between the pharmacological effect and the receptor-binding data, in the case of Bay k 8644. On the other hand, as shown in this study and by DePover et al. (1982, 1983a, 1983b); Sarmiento et al. (1983), Lee and Tsien, (1983), and Schwartz and Triggle, (1984), the pharmacological I$_{50}$ for inhibition of contraction and slow inward current in cardiac muscle by nitrrendipine is much higher than that expected from radioligand-binding studies. Although the reason for this is not yet known, one possibility might be that both the agonist and antagonist bind to and compete for only one single class of high affinity dihydropyridine-binding sites. To explain their opposite pharmacological effects, however, we have to assume that one of those drugs has intrinsic activity and the other drug has no intrinsic activity but acts by displacing the first one. Since the pharmacological activities displayed by both agonist and antagonist on intact tissue are independent of each other, this mechanism would be valid only if an endogenous compound existed that had either calcium agonist or calcium antagonist activity and bound reversibly to the high affinity dihydropyridine-binding sites. This putative endogenous compound would presumably diffuse away during preparation of cardiac sarcolemma. An endogenous factor which modulates calcium channel function physiologically has, in fact, recently been postulated (Schramm et al., 1983a, 1983b).

An alternative possibility is that the dihydropyridines bind to more than one class of binding sites with different affinities in cardiac muscle (DePover et al., 1983b). Bay k 8644, a calcium agonist drug, which has a binding constant and pharmacological ED$_{50}$ in a similar concentration range, may activate calcium channels and exert a positive inotropic effect by binding to the high affinity dihydropyridine-binding sites. Binding of nitrrendipine or other dihydropyridine calcium antagonists to the same high affinity binding sites would inhibit the effect of Bay k 8644, as shown by Schramm et al. (1983a, 1983b), but this may not be related to their negative inotropic effect exerted only at higher concentrations. Schramm et al. (1983a, 1983b) have demonstrated a shift in the Bay k 8644 dose-response curve to the right by pretreatment of isolated guinea pig hearts with 3 nM nifedipine, but the shift in the presence of higher (i.e., 30 and 300 nM) nifedipine concentrations was much less than that which is expected when a pure competitive antagonism exists. Bellemann et al. (1981) reported a ‘low affinity’ dihydropyridine-binding site with a K$_D$ of 67 nM, in guinea pig cardiac membrane fractions with “an unknown pharmacological role.” More recently, Marsh et al. (1983) showed that low affinity nitrrendipine-binding sites also exist in cultured chick embryo ventricular cells, with a K$_D$ of 19 nM, which was comparable to the I$_{50}$ of 23 nM for the negative inotropic effect of this drug. On the basis of these data, Marsh et al. (1983) suggested that the binding of dihydropyridines to these low affinity sites may be related to their negative inotropic effect in the heart. Our data at this point are consistent with this concept. In our scheme, however, the high affinity dihydropyridine-binding site is related to the activation of calcium channels and positive inotropic effects of dihydropyridines and, therefore, is pharmacologically relevant. We assume that those dihydropyridines which have intrinsic activity at the high affinity binding sites, i.e., at putative “agonist receptors,” are calcium agonists. Those dihydropyridines which exert an intrinsic activity at low affinity binding sites, i.e., at putative “antagonist receptors,” are calcium antagonists. It is also possible that some dihydropyridines possess a biphasic action, viz., an agonist effect at low concentrations and an antagonist effect at high concentrations.

### Addendum

After this manuscript was submitted, a paper by Peter Bellemann in FEBS Letters 167: 88–92, 1984, came to our attention showing that Bay k 8644 and dihydropyridine calcium antagonists bind to the same binding site in monolayer cultures of beating myocytes. Another related paper recently appeared by R.A. Janis et al., in Biochem Biophys Res Commun 121: 317–323, 1984, in which $[^3]$HBay k 8644 was found to bind low and high affinity sites on rabbit ventricular microsomes and guinea pig brain synaptosomes.

### Table 1

<table>
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<tr>
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<th>Bay k 8644 (nm)</th>
<th>Nitrendipine (nm)</th>
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<tbody>
<tr>
<td>Atria ED$_{50}$</td>
<td>30.5 ± 9.7 (n=8)</td>
<td>686 ± 92 (n=10)</td>
</tr>
<tr>
<td>Ventricle ED$_{50}$</td>
<td>29.5 ± 8.0 (n=8)</td>
<td>613 ± 109 (n=10)</td>
</tr>
<tr>
<td>Sarcolemma K$_D$</td>
<td>7.44 ± 1.02 (n=4)</td>
<td>0.29 ± 0.025 (n=4)</td>
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ED$_{50}$ indicates the Bay k 8644 concentration which causes a half-maximal increase, and I$_{50}$ indicates the nitrrendipine concentration which produces a half-maximal decrease of force developed in isolated dog atrial and ventricular trabecular muscles. The apparent equilibrium dissociation constants (K$_D$) for the high affinity dihydropyridine-binding sites in isolated canine cardiac sarcolemma were obtained in saturation $[^3]$Hnitrrendipine-binding experiments. Values are means ± SEM.
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INDEX TERMS: [3H]Nitrendipine • Bay k 8644 • Cardiac sarcolemma • Calcium channel • Radioligand binding
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