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SPECIFIC BINDING OF A CALCIUM CHANNEL ACTIVATOR, [^{3}H]BAY k 8644, TO MEMBRANES FROM CARDIAC MUSCLE AND BRAIN

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SUMMARY: BAY k 8644 is a member of a new class of drugs that directly activates Ca^{2+} channels. This 1,4-dihydropyridine was found to bind to both high and low affinity sites on rabbit ventricular microsomes and guinea pig brain synaptosomes. The dissociation constant obtained from Scatchard analysis with [^{3}H]BAY k 8644 was 2 to 3 nM for the high affinity binding site, and the estimated maximal number of binding sites was 0.8 and 0.4 pmol/mg protein for heart and brain membranes, respectively, at 15°C. Competition between nitrendipine and [^{3}H]BAY k 8644 indicated a common high affinity binding site for Ca^{2+} channel activators and antagonists. The results suggest that the 1,4-dihydropyridine Ca^{2+} channel antagonists do not act as simple channel plugs.

Ca^{2+} channel antagonists are drugs that inhibit the movement of Ca^{2+} through Ca^{2+} channels in certain excitable cells (1-7). The most potent drugs of this type, the 1,4-dihydropyridines, have been found to bind specifically and with high affinity to membranes from many cell types (for reviews, see 4 and 7). Correlations between binding affinity and absolute (8) or relative potency (9, 10) for inhibition of smooth and cardiac muscle contraction have supported the hypothesis that the binding site studied mediates the pharmacologic effects. Binding sites with a similar high affinity for 1,4-dihydropyridines are also found in membranes from brain (11-15).

The recent introduction of BAY k 8644 (1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate; Fig. 1), a potent 1,4-dihydropyridine that increases smooth and cardiac muscle contraction (16), provides a new opportunity to examine potential sites of drug binding at the Ca^{2+} channels. BAY k 8644 acts directly on Ca^{2+} channels to increase Ca^{2+} current (17, 18). We now report the high affinity binding of [^{3}H]BAY k 8644 to rabbit ventricular membranes and guinea pig brain synaptosomes. The

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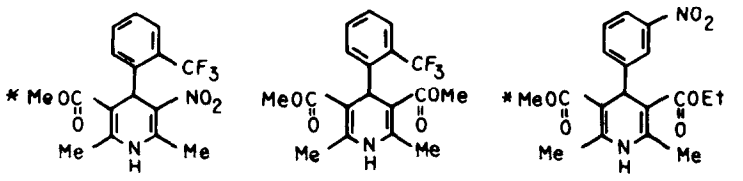

Figure 1. Structural formulas of the Ca^{2+} channel activator BAY k 8644. Also shown are the structures of two potent Ca^{2+} channel antagonists, nitrendipine and a trifluoromethyl analog of nitrendipine. It can be seen that small modifications in drug structure result in cardiovascular effects that are qualitatively different. The positions of the tritium atoms are shown (*) in the radiolabelled compounds.

results suggest that Ca^{2+} channel antagonists do not act by plugging the channel pore.

METHODS

Female rabbits (J. Plummer, Grafton, VT) weighing 1.8-2.4 kg were sacrificed by a single lethal blow to the head, and the hearts removed and quickly placed in 50 mM Tris-HCl, pH 7.4, at 4°C. The atria and fat were quickly removed by dissection. Ventricular myocardium (3.5 g) was minced and homogenized in 35 ml of buffer with a Brinkman Polytron, PT-20 probe at setting 7 for 30 seconds, then repeated after one minute of cooling on ice. The homogenate was centrifuged at 3,000xg for 10 minutes at 4°C. The supernatant was removed and centrifuged at 10,000xg for 10 min and then 100,000xg for 30 min. The microsomal pellet was resuspended in buffer and used within 4 hours. Guinea pig cerebral cortex was homogenized in 0.3 M sucrose (10 volumes) and centrifuged at 1,000xg for 10 min., and the supernatant centrifuged at 17,000xg for 20 min. The pellet (P_2) was gently resuspended in a buffer containing (in mM) NaCl 132; KCl, 5; $MgCl_2$, 1.3; $CaCl_2$ 1.2; glucose, 10; Tris 25; and the pH was adjusted to 7.4 at 25°C with HCl.

Binding was carried out as previously described (8,9), except for the buffer used for incubating synaptosomes, which is described above. Membrane protein, 90-100 µg per 2.5 ml assay volume, was incubated with [^{3}H]BAY k 8644, pH 7.4 at 15°, or 25°C for 60 min. The bound drug was separated from the free by rapid filtration through Whatman GF/B filters, followed by 3 consecutive 3.0 ml washes. Binding experiments were performed under sodium vapor light or under subdued light. Protein was measured using bovine serum albumin as standard (19). Drugs were dissolved in ethanol; this solvent did not affect specific binding at concentrations up to 0.5% v/v. Mean ± S.E. are given in the text and in the figure legends.

Nitrendipine and BAY k 8644 were obtained from Bayer AG. [^{3}H]BAY k 8644 and [^{3}H]nitrendipine (>97% purity; 76-87 Ci/mmol.) were obtained from New England Nuclear, Boston, MA; [^{3}H]BAY k 8644 was custom synthesized.

RESULTS AND DISCUSSION

[^{3}H]BAY k 8644 binding to cardiac membranes was found to be specific and to have a saturable high affinity component (Figs. 2 and 3). Only 40 percent of total binding was displaced by BAY k 8644 at its apparent

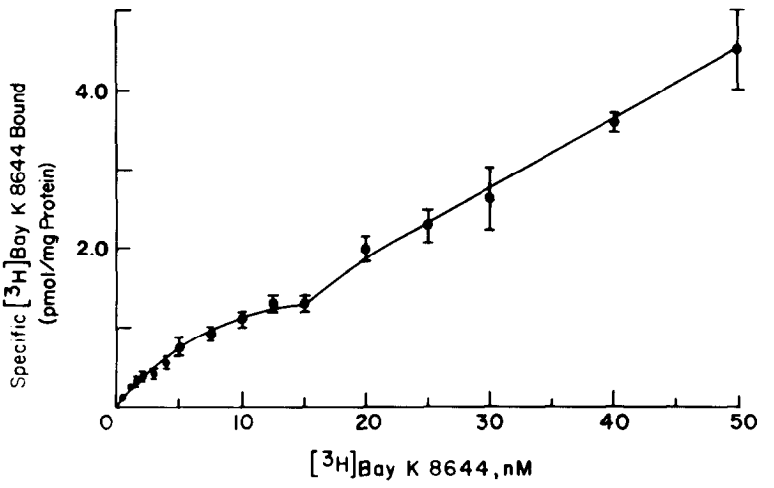

Figure 2. Specific binding of [^{3}H]BAY k 8644 as a function of increasing concentrations of [^{3}H]BAY k 8644. Rabbit ventricular microsomal membranes were incubated at 15°C as described under "Methods", with various concentrations of [^{3}H]BAY k 8644. Nonspecific binding was measured in the presence of 1 μM nonlabelled BAY k 8644 for [^{3}H]-BAY k 8644 concentrations up to 4 nM, and 5 μM nonlabelled BAY k 8644 for [^{3}H]BAY k 8644 concentrations up to 50 nM. Scatchard analysis of the high affinity component gave: K_d = 2.4±0.1 nM, B_{max} = 0.84±0.08 pmol/mg and n_H = 0.97. The data shown are from 5 experiments, each performed in duplicate.

equilibrium dissociation constant (K_d), 2.4 nM. At 3 nM, approximately 20 percent of the total nonspecific binding is to the GF/B glass fiber filters. Two components of [^{3}H]BAY k 8644 specific binding are clearly seen in Fig. 2;

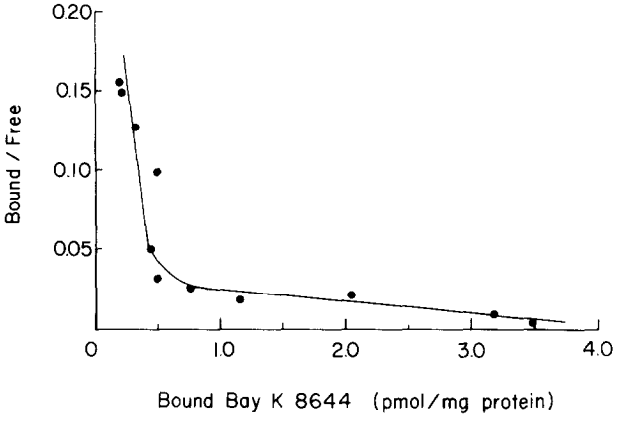

Figure 3. Scatchard plot of specifically bound [^{3}H]BAY k 8644 to heart membranes at 25°C as calculated from the displacement analysis of 1.02 nM [^{3}H]BAY k 8644 by varying concentrations of nonlabelled BAY k 8644. The dissociation constant (K_d) for the high affinity binding site was 2.52 nM and the maximal number of binding sites (B_{max}) was 0.62 pmol/mg of protein for the high affinity site. The apparent Hill slope (n_H) for the high affinity binding site was 1.03 (n=6).

the high affinity component tends to saturate at concentrations around 15 nM. The maximal number of binding sites (B_{max}) for [^{3}H]nitrendipine in these membranes was 0.7 pmol/mg, the same as that for [^{3}H]BAY k 8644. The binding affinity for [^{3}H]BAY k 8644 to guinea pig brain synaptosomes at 15°C was essentially the same as for microsomes from rabbit heart. Data pooled from 6 synaptosomal preparations gave K_d = 3.0±0.58 nM, and a B_{max} of 0.44±0.10 pmol/mg and an apparent Hill coefficient, n_H, of 1.1±0.2.

Nitrendipine displaced [^{3}H]BAY k 8644 from the high affinity binding site; the K_i value for nitrendipine was 0.1 nM, consistent with previous Scatchard results for this ligand (Fig. 4). The apparent Hill slope (n_H) was 0.98. These results are consistent with a common high affinity site of action for the Ca^{2+} channel activator and antagonist. Nitrendipine (1 μM) produced maximal displacement of only 70% of the specifically bound activator; nonspecific binding was defined by 1 μM BAY k 8644. Higher concentrations of nitrendipine were not used in these competition experiments because this would have resulted in inhibitory concentrations of ethanol in those assay tubes used to define nonspecific binding. However, in parallel experiments, the

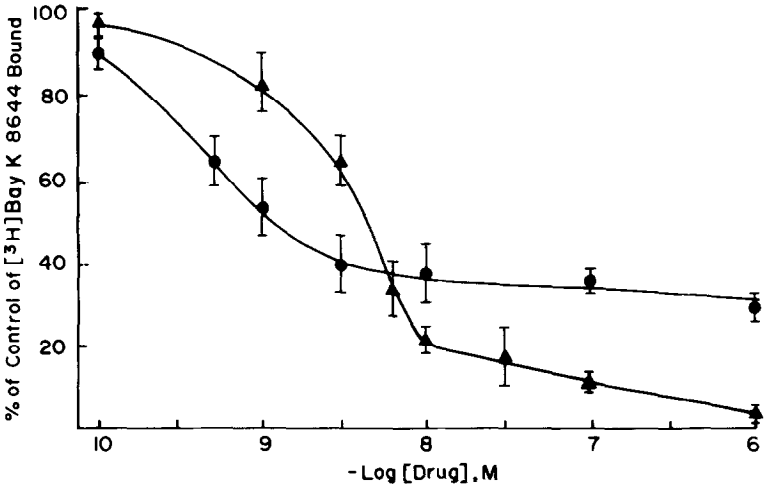

Figure 4. Displacement of [^{3}H]BAY k 8644 (1.02 nM) at 25°C by nonlabelled nitrendipine (●) and BAY k 8644 (▲) from rabbit ventricular microsomal membranes. Nonspecific binding was estimated at each concentration of nonradioactive ligand and was defined as the amount of binding in the presence of 1 μM nonlabelled BAY k 8644. The data shown are from 6 preparations for BAY k 8644 and 5 for nitrendipine, each performed in duplicate.

effect of 5 μM nitrendipine on [^{3}H]BAY k 8644 binding was examined by Scatchard analysis. Nitrendipine (5 μM) completely displaced low (<7 nM) but not high (20 nM) concentrations of [^{3}H]BAY k 8644. Thus, nitrendipine appears to be more effective at competing with BAY k 8644 at the high, than at the low affinity binding site.

Further evidence for a common high affinity site for BAY k 8644 and nitrendipine was seen in the effect of diltiazem on tiapamil-induced inhibition of binding. Tiapamil (10 μM) inhibited both [^{3}H]BAY k 8644 and [^{3}H]nitrendipine binding by 75 to 80%. Diltiazem, 10 to 30 μM, reversed the inhibition of binding of both radioligands caused by tiapamil (to be published elsewhere).

The second component of displaceable binding did not exhibit a clear saturation at 15°C with increasing concentrations of [^{3}H]BAY k 8644 up to 50 nM (Fig. 2). However, preliminary studies indicated that saturation of this site may be more easily seen at 25°C than at 15°C. Therefore, a fixed concentration of [^{3}H]BAY k 8644 was used at 25°C, and saturation of the low affinity binding site with unlabelled BAY k 8644 occurred over the concentration range of 50 to 1000 nM (Fig. 4). Scatchard analysis of these data (Fig. 3) indicated that the K_d was in the range of 50 to 100 nM with a B_{max} of 3 to 4 pmol/mg protein. The modification of this low affinity binding by the same agents (Ca^{2+}, La^{2+}, diltiazem, verapamil) that effect binding at the high affinity site (to be published elsewhere) suggests that this binding represents a relevant site rather than displaceable nonspecific binding.

The dissociation constant of the high affinity binding site for BAY k 8644 in isolated cardiac membranes is at least 10-fold smaller than the apparent ED_{50} values (40-100 nM) for increasing contractility (16) and Ca^{2+} current (18), and is also 10-fold smaller than the recently reported dissociation constant (35 nM) for [^{3}H]BAY k 8644 binding to monolayer cultures of beating myocytes (20). However, Sanguinetti and Kass (21) have found that the effects of both activator and antagonist 1,4-dihydropyridines are voltage-

dependent, and that the high affinity binding site for antagonists may exist on Ca^{2+} channels in the inactivated state. Our working hypothesis is that the binding characteristics of 1,4-dihydropyridines are highly dependent on the various factors that modify the chemical structure (i.e., protein phosphorylation) or state of the Ca^{2+} channel. Therefore, changes in channel state and associated drug binding affinity may occur on membrane isolation.

In conclusion, our results support the hypothesis that the 1,4-dihydropyridine Ca^{2+} channel inhibitors do not act as simple channel plugs because the activator and antagonists share a common high affinity binding site. It is unlikely that BAY k 8644 could activate Ca^{2+} channels and simultaneously occupy a site at which the antagonists sterically plug the channel. It remains to be determined whether the additional binding site for [^{3}H]BAY k 8644 observed in this study reflects binding to a modified Ca^{2+} channel (18).

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