Anti-dihydropyridine Antibodies Exhibit [^{3}H]Nitrendipine Binding Properties Similar to the Membrane Receptor for the 1,4-Dihydropyridine Ca^{2+} Channel Antagonists

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Summary: The [^{3}H]nitrendipine binding properties of anti-dihydropyridine antibodies were characterized and compared with the known [^{3}H]nitrendipine binding properties of the membrane receptor for the 1,4-dihydropyridine Ca^{2+} channel antagonists. Immunization of rabbits with dihydropyridine-protein conjugates resulted in the production of antibodies with high affinity and specificity for the 1,4-dihydropyridine Ca^{2+} channel antagonists. Anti-dihydropyridine antibodies were found specifically to bind [^{3}H]nitrendipine, [^{3}H]nimodipine, [^{3}H]nisoldipine, and [^{3}H]PN200-110 with high affinity, while [^{3}H]verapamil, [^{3}H]diltiazein, and [^{3}H]trifluoperazine were not recognized. [^{3}H]Nitrendipine binding to antidihydropyridine antibodies was characterized by a competitive radioimmunoassay. The average dissociation constant of the [^{3}H]nitrendipine-antibody complex was 0.06 ± 0.02 nM for an antiserum studied in detail, and ranged from 0.01 to 0.24 nM for all antisera studied. The average dissociation constant determined from rate constants of association ($k_1 = 2.7 \times 10^5$ M^{-1} s^{-1}) and dissociation ($k_{-1} = 1.8 \times 10^{-4}$s^{-1}) of [^{3}H]nitrendipinefronianti-dihydropyridine antibodies immobilized on Protein-A Sepharose was 0.66 nM Inhibition of [^{3}H]nitrendipine binding was specific for the 1,4-dihydropyridine Ca^{2+} channel modifiers, and the concentrations required for half-maximal inhibition ranged between 0.25 and 0.90 nM; structurally unrelated Ca^{2+} channel antagonists did not modify [^{3}H]nitrendipine binding to the anti-dihydropyridine antibodies. In summary, anti-dihydropyridine antibodies have been shown to have high affinity and specificity for the 1,4-dihydropyridine Ca^{2+} channel antagonists and to exhibit dihydropyridine binding properties similar to the membrane receptor for the 1,4-dihydropyridine Ca^{2+} channel antagonists. **Key Words:** Nitrendipine – Binding properties – Membrane receptor.

The Ca^{2+} influx via voltage-dependent Ca^{2+} channels initiates and modulates cardiac and smooth muscle contraction (1-3). Drugs that block these voltage-dependent Ca^{2+} channels are used for the treatment of various cardiovascular disorders and are referred to as Ca^{2+} channel antagonists (4-8). Nifedipine, nitrendipine, nisoldipine, and related 1,4-dihydropyridines are the most potent class of Ca^{2+} channel antagonists, and radiolabeled dihydropyridines have been shown to bind with high affinity to membranes isolated from cardiac, skeletal, and smooth muscle (9-13). To better understand the mechanism of action of the dihydropyridine Ca^{2+} channel antagonists, investigators have undertaken the identification of the membrane receptor for these drugs in cardiac and skeletal muscle (14-16), and the isolation and purification of the membrane receptor from enriched sources (i.e., transverse tubular membrane of skeletal muscle) (17-19).

Considerable advances have been made in recent years in the structural and functional characterization of membrane receptors and ion channels using immunelogical probes (20-26), Antibodies have been raised against membrane receptors or ion channels directly by immunization with purified membrane proteins, or indirectly by immunization with anti-ligand antibodies to produce anti-idiotypic antibodies. Anti-idiotypic antibodies have been very useful in studying receptor structure and function, as these antibodies bind to the ligand binding site on the receptor and in some cases can modify receptor function (21). Anti-ligand antibodies (20,21,23,26-29) have been used to study structure-activity relationships between the ligand and antibody, and as specific molecules that can compete with the receptor for the ligand.

We report here the characterization of [^{3}H]nitrendipine binding properties of anti-dihydropyridine anti-

bodies. High-affinity antibodies specific for the 1,4-dihydropyridine Ca^{2+} channel blockers were produced using either a bovine serum albumin-dihydropyridine conjugate or a keyhole limpet hemocyanin-dihydropyridine conjugate. The protein-dihydropyridine conjugates were prepared using an affinity analog ofnifendipine – 1,4-dihydro-2,6-dimethyl-4-(2-isothiocyanatophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester – which can react covalently with free ammo groups in both proteins to form a covalent dihydropyridine-protein conjugate. Anti-dihydropyridine antibodies have been found to bind [3H]nitrendipine with high affinity and specificity. The dihydropyridine binding properties of the anti-dihydropyridine antibodies suggest that the antibodies resemble the membrane receptor for the 1,4-dihydropyridine Ca^{2+} channel blockers with respect to affinity and specificity of 1,4-dihydropyridine binding.

METHODS

Preparation of dihydropyridine-protein conjugates

An affinity analog of nifedipine – 1,4-dihydro-2,6-dimethyl-4-(2-isothiocyanatophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (nifedipine-isothiocyanate) – was used to couple nifedipine covalently to various carrier proteins (i.e., bovine serum albumin, ovalbumin, and keyhole limpet hemocyanin) (see Fig. 1). The carrier protein (10 mg) was first dissolved in 10 ml of 100 mM sodium bicarbonate (pH 9.0) and then incubated with 0.6 mg of dihydropyridine-isothiocyanate (in ethanol) for 24 h at 37°C with shaking in the dark. The final concentration of nifedipine-isothiocyanate and ethanol in the incubation medium was 168 μM and 0.65%, respectively. Incorporation of nifedipine-isothiocyanate was monitored by including [3H]ni-
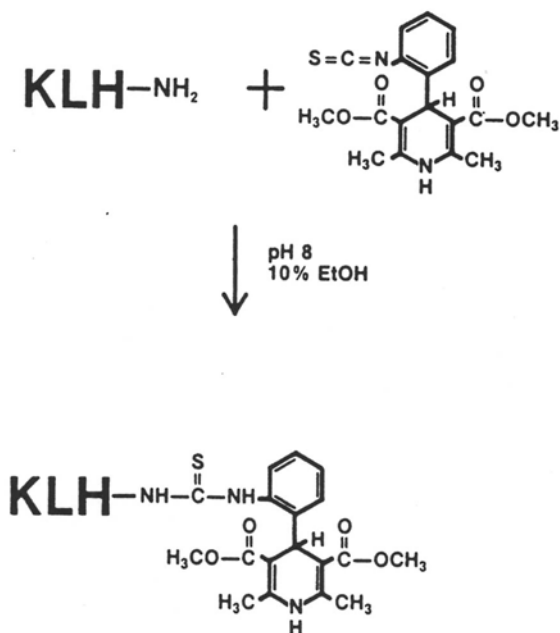
fedipine-isothiocyanate {[3-methyl-3H]-1,4-dihydro-2,6-dimethyl-4-(2-isothiocyanotophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester} (2.5 μCi/ml) during the formation of the conjugate. The [3H]nifedipine-labeled proteins were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis according to Laemmli (30) using 1.5 mm thick gradient slab gels (5-16%, acrylamide). The [3H]nifedipine incorporation was determined by liquid scintillation counting of gel slices as described previously (15).

Immunization and antibody preparation

New Zealand white rabbits were first bled to obtain preimmune sera and then immunized intramuscularly and subcutaneously at multiple sites along the back and legs. Rabbits received 0.5 mg of nifedipine conjugate in Freund's complete adjuvant on day 0, followed by 0.5 mg insults in Freund's incomplete adjuvant every 2-3 weeks. The bovine serum albumin-nifedipine conjugate was used directly or cross-linked using 0.04% glutaraldehyde for 8 h at room temperature before immunization. In either case, the conjugate was emulsified in an equal volume of Freund's adjuvant. The keyhole limpet hemocyanin-nifedipine conjugate was mixed with an equal volume of aluminum hydroxide and phosphate-buffered saline (31), stirred for 1 h at 4°C, and then emulsified in an equal volume of Freund's adjuvant. Rabbits were bled after their third immunization every 2-3 weeks. Blood was withdrawn from the ear vein, allowed to clot overnight at 4°C, and serum was isolated following centrifugation. Either antisera were used directly or antibodies were partially purified from antisera by ammonium sulfate precipitation in the cold for 24 h. The precipitated immunoglobulin fraction was resuspended and desalted by passage through a Pharmacia PD-10 Sephadex G-25 column in the presence of phosphate-buffered saline at 4°C.

[3H]Nitrendipine binding: Protein-A Sepharose assay

A [3H]nitrendipine binding assay was performed in triplicate using 1.5 ml Eppendorf tubes in the dark. Each assay tube contained 150 mM NaCl, 10 mM TRIS (pH 7.2), 1 nM [3H]nitrendipine, 10 μl/ml antiserum, and 50 μl/ml Protein-A Sepharose 4B in a final volume of 1.0ml. Samples were slowly mixed by rotation for 1.5 h in the dark at room temperature. Protein-A Sepharose beads were collected by centrifugation in an Eppendorf centrifuge and then washed three times in 150 mM NaCl, 10 mM TRIS (pH 7.2). The [3H]nitrendipine bound was eluted by the addition of 0.1 M glycine (pH 2.8) and measured by liquid scintillation counting. The [3H]nitrendipine on the Protein-A Sepharose beads represents the [3H]nitrendipine bound to the antibody. Binding of various radiolabeled dihydropyridine Ca^{2+} channel blockers — [3H]nimodipine, [3H]nisoldipine, and [3H]PN 200-110 — and various radiolabeled Ca^{2+} channel blockers or antagonists — [3H]verapamil, [3H]diltiazem, and [3H]trifluoperazine — were also measured using the Protein-A-Sepharose assay.

Affinity and specificity of antidihydropyridine antibodies

A competitive radioimmunoassay was used to determine the specificity and affinity of the anti-dihydropyridine antibodies. Average antibody dissociation constants, antibody specificity, and concentration of specific antibodies in the antiserum were calculated according to the methods of Muller (31). The assay was performed under equilibrium

FIG. 1. Affinity analog of nifedipine used to couple nifedipine covalently to various carrier proteins.

conditions, and a titer test was first performed to determine the antiserum or antibody dilution required for 50% of the added [³H]itrendipine to be bound in the absence of unlabeled nitrendipine. The separation of free [³H]nitrendipine from bound [³H]nitrendipine was achieved by the addition of dextran-coated charcoal to the assay medium.

The [³H]nitrendipine binding was performed in triplicate, and each assay tube contained 1.0 ml of 150 mM NaCl, 10 mM TRIS (pH7.2), 0.1% gelatin, 20 pM [³H]nitrendipine, and the appropriate amount of antibody determined by the titer test. After a 1.0 h incubation in the dark, the samples were incubated on ice for 10 min and then 0.2 ml of dextran-coated charcoal was added to each assay tube. The tubes were shaken and then incubated on ice for 12 min. The charcoal was removed by centrifugation (850 × g) for 8 min and [³H]nitrendipine was measured in 0.8 ml supernatant using a liquid scintillation counter. Controls were run without the addition of antiserum or antibody, and the amount of [³H]nitrendipine in the supernatant after the addition of dextran-coated charcoal was subtracted from the value obtained in the presence of antibody to yield the amount of [³H]nitrendipine bound to antibody. Controls run in the absence of antisera showed that the dextran-coated charcoal was able to remove >95% of the free [³H]nitrendipine from the assay medium.

Inhibition of [³H]nitrendipine binding was measured in the presence of unlabeled nitrendipine (10^{-12}-10^{-7} M), and the percentage of [³H]nitrendipine bound was plotted as a function of the concentration of unlabeled nitrendipine. Nitrendipine standards were prepared by serial dilution in 0.5% bovine serum albumin in 150 mM NaCl, 10 mM TRIS (pH 7.2). The average antibody dissociation constant was calculated from the molar nitrendipine concentration for 50% inhibition of [³H]nitrendipine-antibody binding, the molar [³H]nitrendipine concentration in the assay, and the amount of [³H]nitrendipine bound in the absence of unlabeled nitrendipine.

Antibody specificity was determined by inhibition of [³H]nitrendipine binding in the presence of various unlabeled Ca^{2+} channel drugs or related compounds (10^{-12}-$10^{-4}M$), as described previously for nitrendipine. Half-maximal inhibition was determined from a plot of percentage [³H]nitrendipine bound versus the concentration of unlabeled drug.

Kinetics of [³H]nitrendipine binding to anti-dihydropyridine antibodies

Association and dissociation kinetics of [³H]nitrendipine binding to the antidihydropyridine antibodies were determined using antidihydropyridine antibodies bound to Protein-A Sepharose. An antibody-Protein-A Sepharose complex was formed by incubating the dihydropyridine-specific antisera (2.0ml) with Protein-A Sepharose (1.0ml) overnight at 4°C, followed by extensive washing in 150 mM NaCl, 10 mM TRIS (pH 7.2). Association kinetics were started by the addition of the antibody-Protein-A Sepharose complex to the assay mixture with 1.0 nM [³H] nitrendipine. Aliquots were taken at different times and bound radioactivity was measured following centrifugation, as described previously. Nonspecific binding was measured in the presence of 1.0 μM. nitrendipine. Specific binding was calculated by subtraction of nonspecific binding from total binding. Dissociation kinetics were followed after 60 min of association, at which time the amount of specifically bound [³H]nitrendipine had reached a plateau value. Dissociation

was initiated by the addition of 1.0 μM unlabeled nitrendipine, which was able to displace the [³H]nitrendipine from the anti-dihydropyridine antibodies.

Materials

The [³H]nitrendipine (88 Ci/mmol), [³H]verapamil (85 Ci/mmol), [³H]diltiazem (72 Ci/mmol), 1,4-dihydro-2,6-dimethyl-4(2-isothiocyanatophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester, and [3-methyl-³H]-1,4-dihydro-2,6-dimethyl-4-(2-isothiocyanatophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (76.6 Ci/mmol) were obtained from New England Nuclear, Nitrendipine, nimodipine, nisoldipine. Bay k 8644, [³H]nisoldipine (81.5 Ci/mmol), and [³H]nimodipine (145 Ci/mmol) were generously supplied by Miles Laboratories. The [methyl-³H]PN 200-110 (70-85 Ci/mmol), [³H]azidopine (44.6 Ci/mmol-), and [¹²⁵I]iodipine (2000 Ci/mmol) were generously supplied by Amersham. Nifedipine, verapamil, and diltiazem were generously supplied by Pfizer, Knoll Pharmaceutical Co. and Marion Laboratories, respectively. Ryanodine was obtained from Penick (Lyndhurst, NJ, U.S.A.). Bovine serum albumin (Fraction V), keyhole limpet hemocyanin, dextran (~ 70,000 MW), and trifluoperazine were obtained from Sigma (St. Louis, MO, U.S.A.). Charcoal (Norit A) was obtained from Fisher. Dextran-coated charcoal was prepared by mixing 62.5 mg Dextran-70, 625 mg Norit A charcoal, and 100 ml 150 mM NaCl, 10 mM TRIS HCl, pH 7, 0.1% gelatin. The 4-chloro-l-naphthol, diethyl l,4-dihydro-2,4,6-trimethyl-3,5-pyridine-dicarboxylate, 3,5-diacetyl-l,4-dihydro-2,6-dimethylpyridine, 3,4-dimethoxybenzaidehyde, 2,6-lutidine, and 2,6-dimethylpiperidine were obtained from Aldrich. Protein-A Sepharose 4B was obtained from Pharmacia. All other reagents were of reagent grade quality. Protein was determined by the method of Lowry et al. (33) using the modification of Peterson (34).

RESULTS

Preparation and characterization of dihydropyridine-protein conjugates

The nifedipine-protein conjugates were prepared using an affinity analog of nifedipine — 1,4-dihydro-2,6-dimethyl-4-(2-isothiocyanatophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (nifedipine-isothiocyanate) (14). Incorporation of nifedipine-isothiocyanate into bovine serum albumin was monitored by including [³H]nifedipine-isothiocyanate in the preparation of the bovine serum albumin-dihydropyridine conjugate. The [³H]nifedipine-bovine serum albumin conjugate was analyzed by separation of SDS-polyacrylamide gel electrophoresis, and [³H]nifedipine incorporation was measured by gel slice analysis. Figure 2 shows [³H]nifedipine covalent incorporation into bovine serum albumin. Analysis of the total counts of [³H]nifedipine incorporated into bovine serum albumin showed that ~ 1 mol of [³H]nifedipine was incorporated per mole of bovine serum albumin. The [³H]nifedipine incorporation was also observed for casein and ovalbumin at the same levels. Because of keyhole limpet hemocyanin's high molecular weight, nifedipine incorporation could not be analyzed by SDS gel electrophoresis, but was detected by indirect immunostaining with dihydropyridine-specific anti-
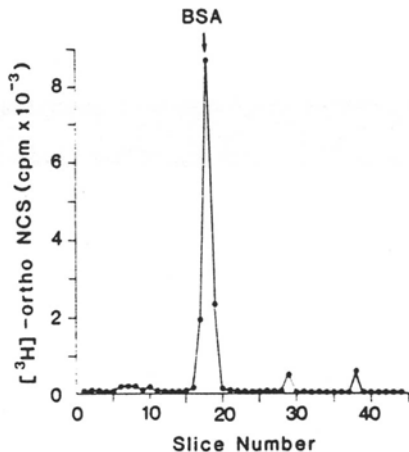
FIG. 2. [³H]Nifedipine incorporation into bovine serum albumiun (BSA). BSA was labeled with ([³H]nifedipine-isothiocyanate ([³H]-ortho NCS) as described in the Experimental Procedures section. [³H]Nifedipine—BSA conjugate (40 μg of protein) was analyzed by SDS—polyacrylamide gel electrophoresis. ([³H]Nifedipine incorporation was measured by liquid scintillation counting of 2 nm gel slices, which were solubilized with hydrogen peroxide. The position of BSA is indicated by the arrow.

serum. The structure of the KLH-dihydropyridine conjugate is given in Fig. 1.

[³H]Nitrendipine binding properties of anti-dihydropyridine antibodies

The binding of free [³H]nitrendipine (1.0 nM) to the antidihydropyridine antibodies in postimmune serum was tested using the Protein-A Sepharose immunoprecipitation technique. Figure 3 shows that in the presence of antiserum, the amount ofpHjnitrendipine bound to the antibody-Protein-A Sepharose complex was > 62,000 cpm or 62% of the total [³H]nitrendipine

in the assay medium. In the absence of added antiserum, or in the presence of preimmune serum, the amount of [³H]trendipine bound to the Protein-A Sepharose was < 100 cpm. The addition of 1.0 μM nitrendipine to the assay with antiserum reduced the [³H]nitrendipine bound to 5900 cpm. Thus, the anti-dihydropyridine antibodies specifically bound 90% of the [³H]trendipine, or 56.1 fmol of [³H]nitrendipine per microliter of antisera.

Radioligand binding to antidihydropyridine antibodies

Figure 4 shows the binding of various radiolabeled Ca^{2+} channel blockers, or Ca^{2+} antagonists, at a 1.0 nM concentration to the anti-dihydropyridine antibodies measured by the Protein-A Sepharose technique. The anti-dihydropyridine antibodies were able to bind [³H]nitrendipine, [³H]nimodipine, [³H]nisoldipine, and [³H]PN 200-110, which are all radiolabeled 1,4-dihydropyridine Ca^{2+} channel blockers. The [³H]verapamil and [³H]diltiazem, which are radiolabeled Ca^{2+} channel blockers, and [³H]trifluoperazine, which is a radiolabeled Ca^{2+} antagonist (calmodulin inhibitor), were not recognized by the antidihydropyridine antibodies. The antidihydropyridine antibodies bound 40-60 fmol 1,4-dihydropyridine Ca^{2+} channel blocker per microliter of antiserum. The anti-dihydropyridine antibodies were also able to bind [³H]azidopine and [³H]iodipine (not shown), which are radiolabeled 1,4-dihydropyridines that have complex side chains.

Determination of affinity of anti-dihydropyridine antibodies by competitive radioimmunoassay

Affinity of the anti-dihydropyridine antibodies for [³H]nitrendipine, and the concentration of specific anti-
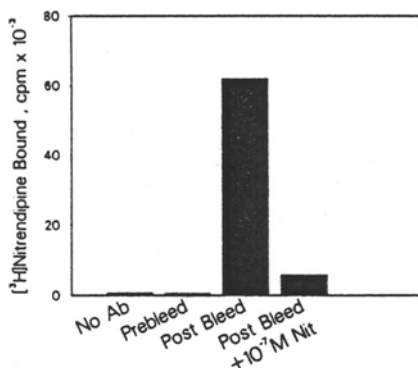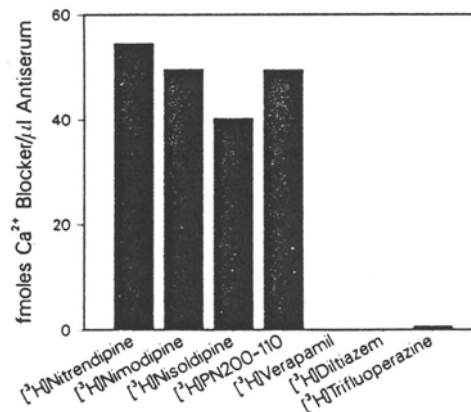

FIG. 3. [³H]Nitrendipine binding to the antidihydropyridine antibodies: Protein-A Sepharose assay. Binding of free [³H]nitrendipine to the antidihydropyridine antibodies was performed using the Protein-A Sepharose assay, as described in Experimental Procedures section. Each assay tube contained 1.0 ml of 150 mM NaCl, 10nM TRIS (pH 7.2), 1.0 nM [³H]nitrendipine, and 50 μl/ml of Protein-A Sepharose 4B beads. [³H]Nitrendipine binding (*solid bars*) was determined in triplicate with the standard deviation of bound [³H]nitrendipine <2%. Samples contained no antibody (No Ab), 10 μl of preimmune sera (Prebleed). 10 μl of postimmune sera (Post Bleed), and 10 μl of postimmune sera plus 10⁻⁷ M nitrendipine (Post Bleed + 10⁻⁷ M Nit).

FIG. 4. Radiolabeled Ca^{2+} channel blocker or Ca^{2+} antagonist binding to the antidihydropyridine antibodies. Binding of various radiolabeled Ca^{2+} channel blockers – [³H]nitrendipine, [³H]nimodipine, [³H]nisoldipine, [³H]PN 200-110, [³H]verapamil, and [³H]diltiazem — or Ca^{2+} antagonists ([³H]trifluoperazine) to the antidihydropyridine antibodies was measured using the Protein-A Sepharose 48 assay, as described in the Experimental Procedures section in the presence of 1.0 nM of the radiolabeled drugs. The total amount of radioligand bound (*solid bars*) was determined and expressed as fmoles Ca^{2+} blocker bound per microliter of antiserum. Standard deviations of the bound radioligand were <2%.
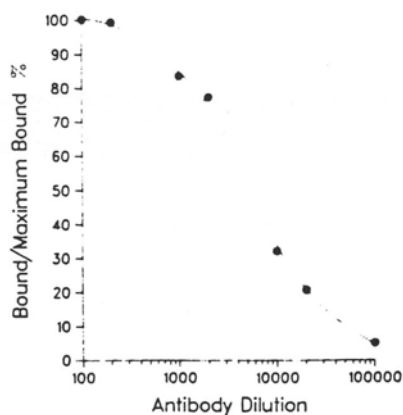
FIG. 5. Titer test for [^{3}H]nitrendipine binding to anti-dihydropyridine antibodies. [^{3}H]Nitrendipine binding was determined, as described in the Experimental Procedures section for the competitive radioimmunoassay. Each assay tube contained 1.0 ml of 150 m*M* NaCl, 10 m*M* TRIS (pH 7.2), 0.1 % gelatin, 20 p*M* [^{3}H]nitrendipine. and various dilutions of an immunoglobulin fraction containing antidihydropyridine antibodies. Separation of free [^{3}H]nitrendipine and antibody-bound [^{3}H]nitrendipine was achieved by the addition of dextran-coated charcoal. Percent of [^{3}H]nitrendipine bound was plotted as a function of antibody dilution. In this experiment, the liter of the antibody was 1:5000.

FIG. 6. Displacement of specifically bound [^{3}H]nitrendipine by untabeled nitrendipine. A competitive radioimmunoassay for [^{3}H]nitrendipine binding was performed according to the conditions of Müller (31) to determine the average antibody dissociation constant. [^{3}H]Nitrendipine binding was measured using the dextran-coated charcoal assay, as described in the Experimental Procedures section in the presence of 20 p*M* [^{3}H]nitrendipine, a 1:5000 dilution of antidihydropyridine antibody, and various concentrations of unlabeled nitrendipine (10^{-12}-10^{-7} *M*). The data shown are from a representative experiment with the standard deviation for each point <2%. In this experiment, the average antibody dissociation constant and the concentration of specific antidihydropyridine antibodies were determined to be 0.06 n*M* and 0.3 mg/ml, respectively.

dihydropyridine antibodies in antisera, have been determined using a competitive radioimmunoassay, according to the method of Müller (31). Before each competitive radioimmunoassay, a titer test was performed on each antiserum to determine the dilution required for 50% of the added [^{3}H]nitrendipine to be bound in the absence of unlabeled nitrendipine. Figure 5 shows the results of a titer test performed in the presence of 20 p*M* [^{3}H]nitrendipine. The titer for the various antisera produced ranged from 1:1000 to 1:145,000.

Figure 6 shows the results of a typical competitive radioimmunoassay used for the determination of the affinity of anti-dihydropyridine antibodies for [^{3}H]nitrendipine and for the concentration of specific antidihydropyridine antibodies in the antiserum. The average apparent dissociation constant (K_d) of the [^{3}H]nitrendipine-antibody complex was 0.06 ± 0.02 n*M* for this antiserum, which was studied in detail. The average dissociation constant for all antisera produced ranged from 0.01 to 0.24 n*M*. Table 1 shows the average apparent dissociation constant and concentration of specific anti-dihydropyridine antibodies for antisera prepared from two rabbits and one sheep. An examination of antisera from the same animal immunized over several months has shown that the average dissociation constant did not change significantly, while the titer or specific antibody concentration increased with additional immunizations.

Association and dissociation kinetics of [^{3}H]nitrendipine binding

Kinetics of association and dissociation of [^{3}H]nitrendipine to immobilized anti-dihydropyridine antibodies were determined using a modified Protein-A
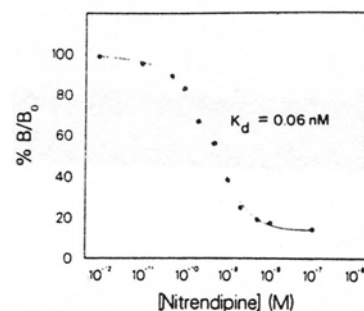
Sepharose assay. Typical kinetics of association of [^{3}H]nitrendipine to the antibody-Protein-A Sepharose complex are presented in Fig. 7A. The semilogarithmic representation of these results, as shown in Fig. 7A, is linear, as expected for a pseudo-first-order reaction. Kinetics of dissociation of [^{3}H]nitrendipine from the antibody-Protein-A Sepharose complex following the addition of excess unlabeled nitrendipine, are present in Fig. 7B. The semilogarithmic representation of these results, as shown in Fig. 7B, is also linear, as expected for first-order kinetics. Kinetic constants calculated from Figs. 7A and 7B were $k_1 = 2.7 \times 10^5 M^{-1} s^{-1}$ and $k_{-1} = 1.8 \times 10^{-4} s^{-1}$. The dissociation constant calculated from the kinetic data is $K_d = k_{-1}/k_1 = 0.66$ n*M*.

TABLE 1. *Average dissociation constant and concentration of specific anti-dihydropyridine antibodies[a]*

Animal	$K_d{}^b$ (n*M*)	[Ab]^c (mg/ml)
Rabbit R	0.05 ± 0.01	0.11-0.25
Rabbit I	0.02 ± 0.01	0.08-1.14
Sheep A	0.18 ± 0.05	0.19-2.13

[a] The average dissociation constant and concentration of specific anti-dihydropyridine antibodies were determined using a competitive radioimmunoassay, as described in the Experimental Procedures section.
[b] Dissociation constants given are the average ± standard deviation for antiserum prepared from three different bleeds from the same animal.
[c] Concentration of specific anti-dihydropyridine antibodies [Ab] given are the lower and upper values obtained for antisera from different bleeds from the same animal.
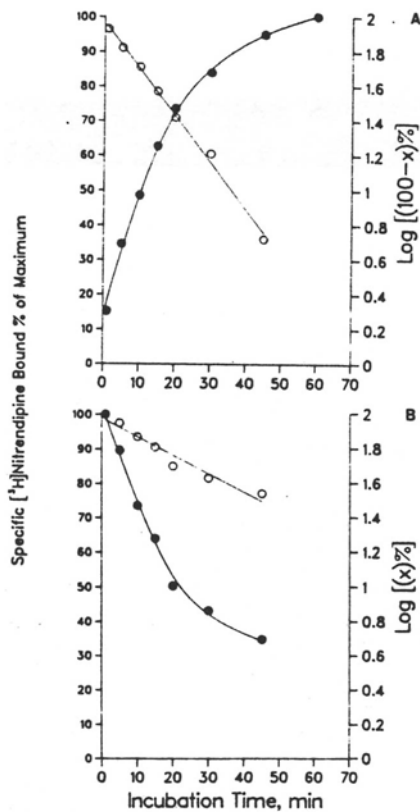
FIG. 7. Association and dissociation kinetics for the binding of [³H]nitrendipine to the anti-dihydropyridine antibodies. [³H]Nitrendipine binding was determined using anti-dihydropyridine antibodies complexed with Protein-A Sepharose, as described in the Experimental Procedures section. **A**: Association kinetics of [³H]nitrendipine (1.0 n*M*) binding at 4°C to the antibody-Protein-A Sepharose complex. Nonspecific binding (< 10%) in the presence of 1.0 μM unlabeled nitrendipine was subtracted to yield specific binding. Time course of [³H]nitrendipine binding (●) and pseudo-first-order representation of the association kinetic data (○), ×, the percentage of maximal [³H]nitrendipine bound to the antibody—Protein-A Sepharose complex. **B**: Dissociation kinetics of [³H]nitrendipine binding at 4°C. After maximal [³H]nitrendipine binding was reached, dissociation of [³H]nitrendipine was initiated by the addition of excess (1.0 μM) nitrendipine. Time course of dissociation (●) and first-order representation of the kinetic data (○).

TABLE 2. *Inhibition of [³H]nitrendipine-antibody binding by various Ca^{2+} channel antagonists[a]*

Compound	% Inhibition at 10^{-7} M[b]	IC_{50} (nM)[c]
Dihydropyridines		
Nitrendipine	85.6 ± 0.4	0.60
Nisoldipine	85.9 ± 0.7	0.34
Nifedipine	85.9 ± 0.7	0.25
Nimodipine	82.9 ± 0.6	0.90
Bay k 8644	83.4 ± 4.4	0.60
Ca^{2+} Antagonists		
Diltiazem	5.7 ± 2.2	>1000
Verapamil	2.5 ± 1.6	>1000
Trifluoperazine	<1.0	>1000
Ryanodine	<1.0	>1000

[a] [³H]Nitrendipine binding to anti-dihydropyridine antibodies was performed using the dextran-coated charcoal assay, as described in the Experimental Procedures section. Inhibition of [³H]nitrendipine binding to the dihydropyridine-specific antibodies was measured by the addition of various unlabeled drugs or reagents (10^{-12}-10^{-4} M) in the presence of 20 p*M* [³H]nitrendipine.
[b] Inhibition of [³H]nitrendipine binding at 10^{-7} M of unlabeled drug or reagent is the average $\pm$ standard deviation.
[c] Half-maximal inhibition (IC_{50}) was determined from a plot of percent [³H]nitrendipine bound versus concentration of drug or reagent.

Specificity of anti-dihydropyridine antibodies

The specificity of the anti-dihydropyridine antibodies has been examined using various Ca^{2+} channel modifiers and related compounds in the presence of 20 p*M* [³H]nitrendipine. Figure 8 shows the displacement of specifically bound [³H]nitrendipine by several Ca^{2+} channel blockers and one Ca^{2+} channel activator. Increasing concentrations of 1,4-dihydropyridine Ca^{2+} channel blockers (nifedipine, nisoldipine) gradually reduced the specific binding of [³H]nitrendipine to the anti-dihydropyridine antibodies. Inhibition of [³H]nitrendipine binding in the presence of 10^{-7} M dihydropyridine ranged between 83 and 86% (Table 2). Half-maximal inhibition (IC_{50}) of [³H]nitrendipine binding by various 1,4-dihydropyridines required between 0.25 and 0.90 n*M* (Table 2). Bay k 8644, a 1,4-dihydropyri-

dine Ca^{2+} channel activator (35), was also able to displace specifically [³H]nitrendipine from the antibody binding site, and half-maximal inhibition required 0.60 nM. Structurally unrelated Ca^{2+} channel blockers, verapamil and diltiazem, did not inhibit the binding of [³H]nitrendipine to the anti-dihydropyridine antibodies (Fig. 8, Table 2). Ryanodine, a possible intracellular Ca^{2+} channel blocker, also did not inhibit [³H]nitrendipine binding (Fig. 8, Table 2). Therefore, the antidihydropyridine antibodies are specific for the 1,4-dihydropyridine Ca^{2+} channel blockers, but other Ca^{2+} channel blockers, or Ca^{2+} antagonists, do not allosterically regulate [³H]nitrendipine binding to the antibodies.

DISCUSSION

The production of antibodies, specific for the 1,4-dihydropyridine Ca^{2+} channel antagonists, required the synthesis of an immunogenic dihydropyridine-protein conjugate. An affinity analog of nifedipine, nifedipine-isothiocyanate, has been used to prepare dihydropyridine-protein conjugates that have the essential moieties of nifedipine intact and available to the immune system. Preliminary labeling experiments in our laboratory with [³H]nifedipine-isothiocyanate have indicated that at high concentrations and high pH this analog was able to incorporate nonspecifically into several different proteins. Presumably, the nifedipine-isothiocyanate binds to low-affinity sites in these proteins and then reacts with a free amino group in the

FIG. 8. Displacement of [³H]nitrendipine binding by unlabeled Ca^{2+} channel drugs. [³H]Nitrendipine binding was determined using the dextran-coated charcoal assay, as described in the Experimental Procedures section and Fig. 7. Inhibition of [³H]nitrendipine (20 pM) binding was measured in the presence of increasing concentratipns of the following: nifedipine, nimodipine, and Bay k 8644(10^{-12}-10^{-7} M). and diltiazem. verapamil, and ryanodine (10^{-12}-10^{-4} M).
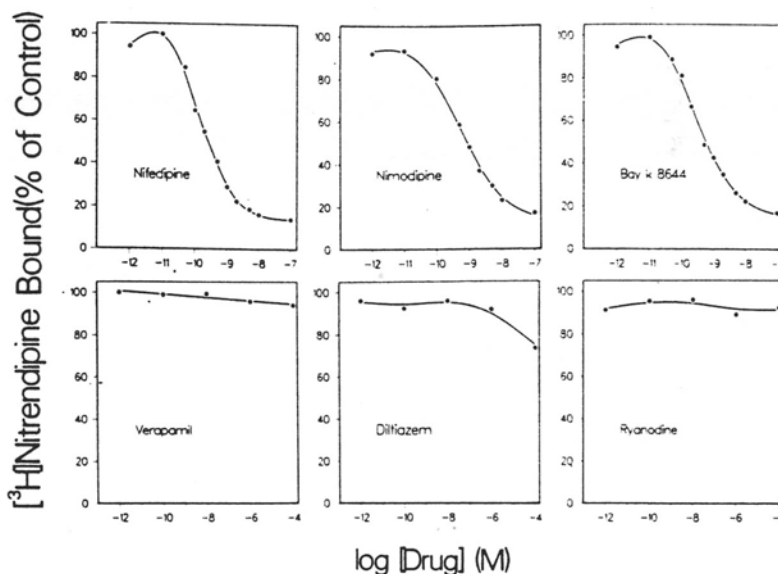
protein to form a covalent nifedipine-protein conjugate. We have optimized the conditions for the low-affinity nifedipine-isothiocyanate labeling and used nifedipine-isothiocyanate to couple ~ 1 mol nifedipine covalently to 1 mol of carrier protein.

The ability of anti-dihydropyridine antibodies in anti-Serum to bind free [³H]nitrendipine was demonstrated by the Protein-A Sepharose assay. This assay required only microliters of antiserum and had a very high signal to noise ratio. In addition, a positive result in this assay indicates that the [³H]nitrendipine binding activity in antiserum is due to immunoglobulins that bind to Protein-A. The responses of 10 rabbits immunized with various dihydropyridine-protein conjugates were monitored using both the immunoblot assay and the Protein-A Sepharose assay. All 10 rabbits produced anti-dihydropyridine antibodies in a range of 10-12 weeks, and their production rose steadily over the initial 20-30 weeks. Detectable anti-dihydropyridine antibodies were present as early as 4 weeks after the initial immunization using keyhole limpet hemocyanin-nifedipine conjugates (see Fig. 1). The concentration of specific anti-dihydropyridine antibodies in antiserum was determined by the method of Müller (31), and ranged from 0.08 to 2.13mg/ml. Repeated immunization of the same animal over 1 year has been shown to increase the concentration of specific anti-dihydropyridine antibodies, while not significantly changing the dissociation constant of the antibodies for [³H]nitrendipine. The concentration of antibodies to the carrier protein was maintained at a relatively low level by changing the carrier protein in the dihydropyridine conjugate every several months.

The dihydropyridine binding properties of the anti-dihydropyridine antibodies, and the membrane receptor for the 1,4-dihydropyridine Ca^{2+} channel blockers, have been compared with respect to affinity and specificity. To calculate the affinity and specificity of the

anti-dihydropyridine antibodies for [³H]nitrendipine, we have used a competitive radioimmunoassay according to the method of Müller (31). Most methods for the determination of affinity constants cannot be applied to antibodies from serum because there is a heterogenous population of antibodies with different affinities. Müller's method requires that the competitive radioimmunoassay be performed under equilibrium conditions, that the antibody concentrations be adjusted to 40-70% tracer binding in the absence of inhibitor, and that the separation technique must almost completely separate bound from free tracer. The competitive radioimmunoassay for [³H]nitrendipine binding to anti-dihydropyridine antibodies that we have developed using dextran-coated charcoal meets all of the aforementioned requirements. The average dissociation constants of the [³H]nitrendipine-antibody complex, determined using the competitive radioimmunoassay, ranged from 0.01 to 0.24 nM for all antisera studied, and are essentially in the same range as that measured for the membrane receptor for the 1,4-dihydropyridine Ca^{2+} channel blockers (9-13). The average dissociation constant calculated from the kinetic data was 0.66 nAf, which is higher than that determined using the competitive radioimmunoassay, but still within the range of that measured for the membrane receptor. Thus, the anti-dihydropyridine antibodies, like the membrane receptor, have high affinity for [³H]nitrendipine. The high affinity of the anti-dihydropyridine antibodies also has been demonstrated by their ability to compete with the membrane receptor for [³H]nitrendipine (K.P. Campbell, unpublished observation).

The specificity of the anti-dihydropyridine antibodies, i.e., the cross-reactivity of antibodies with compounds structurally related to the hapten (nifedipine) used for immunization, has been examined using the competitive radioimmunoassay. In this assay, the competition of [³H]nitrendipine and various Ca^{2+}

channel modifiers for the [³H]nitrendipine antibody binding sites was measured. The specificity of the antibodies was then determined by comparing the concentration required for half-maximal inhibition of [³H]nitrendipine-antibody binding. As shown in Table 2, significant cross-reactivity occurred only with other 1,4-dihydropyridines (nifedipine, nisoldipine, and nimodipine). Concentrations of 1,4-dihydropyridine Ca^{2+} channel blockers required for half-maximal inhibition of [³H]nitrendipine binding ranged between 0.25 and 0.90 nM. Bay k 8644, a Ca^{2+} channel activator (35), was also able to displace specifically [³H]nitrendipine from the antibody, and required 0.60 nM for. half-maximal inhibition. Thus, the antidihydropyridine antibodies mimic the binding characteristics of the membrane receptor for the 1,4-dihydropyridines, with respect to the inhibition of high-affinity [³H]nitrendipine binding by the various 1,4-dihydropyridine Ca^{2+} channel modifiers.

The binding specificity of the anti-dihydropyridine antibodies was also examined using various [³H]-Ca^{2+} channel blockers in the Protein-A Sepharose assay. The anti-dihydropyridine antibodies bound all the [³H]-1,4-dihydropyridine Ca^{2+} channel blockers with high affinity. Structurally unrelated Ca^{2+} channel blockers, or Ca^{2+} antagonists, were not bound by the antibodies. Therefore, the anti-dihydropyridine antibodies bind [³H]-1,4-dihydropyridines in a manner similar to that of the membrane receptor (4-8, 13); but, unlike the membrane receptor, other Ca^{2+} channel blockers are not bound by the antibodies.

The antidihydropyridine antibodies and the 1,4-dihydropyridine membrane receptor have been found to differ with respect to allosteric modification of high-affinity [³H]nitrendipine binding. Structurally unrelated Ca^{2+} channel blockers (verapamil and diltiazem), which are able to regulate allosterically the binding of the 1,4-dihydropyridines to the membrane receptor, did not modify the binding of [³H]nitrendipine to the antibodies. Divalent cation chelation, which is able to inhibit the binding of the 1,4-dihydropyridines to the membrane receptor, also did not modify the binding of [³H]nitrendipine to the antibodies. Therefore, unlike the membrane receptor, the antidihydropyridine antibodies are not allosterically regulated by other Ca^{2+} channel blockers or divalent cation chelation.

It is evident from the data presented that the antidihydropyridine antibodies have a high affinity and specificity for the 1,4-dihydropyridine Ca^{2+} channel blockers. The dihydropyridine binding characteristics of the anti-dihydropyridine antibodies compare favorably with the binding characteristics of the membrane receptor for the 1,4-dihydropyridines and suggest that these antibodies will be very useful in the production of anti-idiotypic antibodies to the dihydropyridine membrane receptor. The ability of the anti-dihydropyridine antibodies to recognize nifedipine, which is covalently attached to protein, suggests that these antibodies will be useful in the immunoprecipitation and immuno-

localization of nifedipine-labeled receptors. Finally, because the anti-dihydropyridine antibodies bind the Ca^{2+} channel antagonists with specificities and affinities similar to those of the membrane receptor, they will be very useful in the development of specific radioimmunoassays for the detection of 1,4-dihydropyridine Ca^{2+} channel antagonists in human plasma.

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