Affinity Purification of Antibodies Specific for 1,4-Dihydropyridine Ca^{2+} Channel Blockers

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High-affinity antibodies specific for the 1,4-dihydropyridine Ca^{2+} channel blockers have been produced in sheep and affinity purified using a dihydropyridine-Sepharose affinity column. Dihydropyridine-Sepharose affinity matrix was synthesized by reaction of aminohexyl-Sepharose with an affinity analogue of nifedipine, dimethyl l,4-dihydro-2,6-dimethyl-4-(2-isothiocyanatophenyl)-3,5-pyridinedicarboxylate. Residual amine groups were then blocked by carbodiimide-catalyzed acetylation. [^{3}H]Nitrendipine-binding activity in serum was specifically absorbed by the dihydropyridine-Sepharose affinity column. The bound antibody was eluted with diethylamine (pH 11.5) in 10% dioxane or with a low-affinity dihydropyridine ligand (diethyl l,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate), pH 7.4. Thirty-six milligrams of highly pure IgG antibody, as demonstrated by sodium dodecyi sulfate-gel electrophoresis, was isolated from 50 ml hyperimmune sheep serum. The affinity-purified anti-dihydropyridine antibodies have been shown to have high affinity (K_d~0.1 nM) and specificity for the 1,4-dihydropyridine Ca^{2+} channel blockers and, therefore, exhibit dihydropyridine-binding properties similar to the membrane receptor for the 1,4-dihydropyridine Ca^{2+} channel blockers. Immunoblot staining of anazidopine-bovine serum albumin conjugate with affinity-purified anti-dihydropyridine antibodies demonstrated that the anti-dihydropyridine antibodies recognize the 1,4-dihydropyridine Ca^{2+} channel blockers when covalently coupled to protein and, therefore, should be useful in the identification and purification of receptors covalently labelled with 1,4-dihydropyridine Ca^{2+} channel blockers. (*Circulation Research* 1987;61(suppl I):I-37-I-45)

Influx of Ca^{2+} through voltage-dependent Ca^{2+} channels is important in mediation of contraction in cardiac and smooth muscle.[1-3] Numerous structurally unrelated groups of organic molecules modify conductance of voltage-dependent Ca^{2+} channels[4-8]; the most potent class is 1,4-dihydropyridines.[9-13] Various members of this class are capable of decreasing (e.g., nitrendipine, nifedipine, orPN 200-110) or increasing (e.g., Bay K 8644 or CGP 28392) conductance of voltage-dependent Ca^{2+} channels.[8-14] Specific high-affinity receptors for the dihydropyridine class have been described in cardiac, smooth, and skeletal muscle membranes[19-13] as well as in nerve and brain membranes.[15,18]

To determine the functional significance of the dihydropyridine receptors and the relation of the receptors to Ca^{2+} channels in various tissues, it is useful, if not necessary, to develop immunologic probes to the dihydropyridine receptor. Immunologic probes have proven invaluable in the localization, purification, and determination of membrane protein structure and function.[19-25] Antiidiotypic antibodies, produced by immunization with antiligand antibodies, are particularly interesting immunologic probes because they may be directed against the ligand-binding site and may affect receptor function.[19,26] Antihapten antibodies have been used in the identification of endogenous analogues of drugs or toxins[27,28] and for reversal of drug effects by specific competition with receptors for the hapten.[29,30] Antibodies directed against ligands have also proven useful in the identification and localization of ligand-labelled receptors.[31]

As a step toward development of antibody probes to the dihydropyridine receptor, high-affinity antibodies specific for the 1,4-dihydropyridine Ca^{2+} channel blockers have been produced.[32] For these antibodies to be useful in identification, purification, and localization of receptors labelled with 1,4-dihydropyridine Ca^{2+} channel blockers and for production ofantiidiotypic antibodies directed against the membrane dihydropyridine receptor, an affinity-purified preparation was desirable. In this study, the synthesis of a dihydropyridine-affinity matrix and its use in purification of specific, high-affinity anti-dihydropyridine antibodies is reported; particular emphasis is focused on demonstrating that the affinity-purified antibodies resemble the membrane receptor for the 1,4-dihydropyridine Ca^{2+} channel blockers in their binding characteristics for the 1,4-dihydropyridines and that the affinity-purified antibodies were able to recognize the 1,4-dihydropyridines that were covalently attached to protein. The methods described should also prove useful in purification of the membrane receptor for the 1,4-dihydropyridine Ca^{2+} channel blockers.

Materials and Methods

Synthesis of Dihydropyridine-Sepharose

Figure 1 illustrates the synthesis of the affinity matrix. All steps were performed in darkness or in dim, indirect lighting. In 10 ml 95% ethanol, 1-5 mg nifedi-

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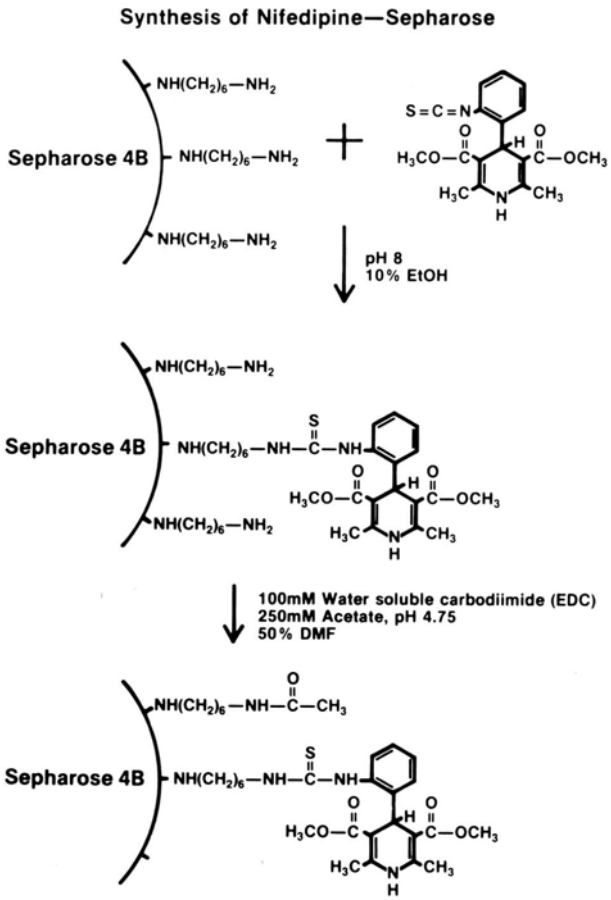

FIGURE 1. *Synthesis of dihydropyridine-Sepharose affinity matrix as described in "Materials and Methods." Nifedipine-isothiocyanate (1–5 mg) was covalentty coupled to aminohexyl-Sepharose 4B (pH 8–-10) in 10% ethanol. After extensive washing, remaining primary amine groups were acetylated using the water-soluble carbodiimide, l-ethyl-3-(3-dimethylaminopropyl)carbodiimide, in 50% dimethylformamide. Washed affinity matrix contained 75–300 nmol nifedipinelml gel, depending on the conditions used.*

pine-isothiocyanate[1] (1,4-dihydro-2,6-dimethyl-4-(2-isothiocyanatophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester) was added to 5 g preswollen and washed aminohexyl-Sepharose 4B in 100 ml 20 mM $NaHCO_3$, pH 8.0–10.0. The mixture was shaken at 37° C overnight in the dark. Then, the dihydropyridine-Sepharose 4B was washed three times with 100 ml 1 M Tris-HCl buffer (pH 7.5) followed by exhaustive washing with 150 mM NaCI and 10 mM Tris-HCl (pH 7.2). To block residual amine groups, the matrix was washed with water followed by 50% dimethyl formamide (vol/vol) and 250 mM sodium acetate (pH 4.75). Finally, 50% dimethyl formamide and 250 mM sodium acetate (pH 4.75) was added to a final volume of 5 ml/ml dihydropyridine-Sepharose 4B, and l-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (water-soluble carbodiimide) was added slowly as a powder to a final concentration of 100 mM. The mixture was allowed to react overnight at 30° C with mixing, and the gel was then washed extensively with 50 mM sodium phosphate (pH 7.4) and 150 mM NaCl.

The amount of nifedipine-isothiocyanate incorporated into the affinity matrix was monitored by including [3-methyl-^{3}H]-1,4-dihydro-2,6-dimethyl-4-(2-isothiocyanatophenyl) - 3,5 - pyridinedicarboxylic acid dimethyl ester ([^{3}H]nifedipine-isothiocyanate) during the coupling. Measured quantities of washed, suspended affinity matrix were pipetted into Budget Solve scintillation fluid (Research Products International, Mt. Prospect, Ill.) and counted in a scintillation counter. The degree of derivatization could be controlled by varying the nifedipine-isothiocyanate concentration and the pH of the coupling mixture.

Production of Anti-Dihydropyridine Antibodies

An affinity analogue of nifedipine, nifedipine-isothiocyanate, was coupled covalently to bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) as described previously.[32] A female sheep was initially injected with 5 mg i.m. BSA-nifedipine conjugate in Freund's complete adjuvant. Booster injections of 5 mg i.m. BSA-nifedipine conjugate in Freund's incomplete adjuvant were made after 2, 4, and 8 weeks. Booster injections were switched to KLH-nifedipine for subsequent boosts at 1-month intervals. The sheep was bled from the jugular vein 3 weeks after the third injection and at 2-week intervals thereafter, 1 week after boosting or as needed. Sheep blood was collected in a flask containing coils of aluminum wire and shaken vigorously for 15 minutes. The flask was then cooled to 0° C and allowed to stand for 1 hour. The fluid portion was decanted off and centrifuged, and the clear serum was collected. [^{3}H]Nitrendipine-binding activity, detectable in all postimmune bleeds, rose to a high titer after 16 weeks.

Affinity Chromatography of Anti-Dihydropyridine Antibodies

Dihydropyridine-Sepharose 4B was poured into a column and washed with 15-column volumes of 50 mM sodium phosphate (pH 7.4) and 150 mM NaCl. Except as noted, serum (10-20-column volumes) was cycled through the column three times from the bottom at 3–4 ml/hr at 4° C using a peristaltic pump. The column was then washed with 50 mM NaH_2PO_4 (pH 7.4) and 1 M NaCl until absorbance at 280 nm returned to baseline. The column was eluted with one of three solutions: The first successful elutions were performed with 50 mM diethylamine (pH 11.5) and 0.5 M NaCl in 10% 1,4-dioxane (vol/vol). Later elutions were performed with 50 mM NaH_2PO_4 (pH 7.4), 1 M NaCl, and $1–2 \times 10^{-4}$ M diethyl l,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylatein 10% 1,4-dioxane or with 50 mM NaH_2PO_4 (pH 7.4), 0.5 M NaCl, and 10^{-4} M diethyl l,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate. Basic elutions were performed at 0–4° C while elutions with diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate were performed at room temperature. Typically, fractions of one-half-column volume were collected and subjected to gel chromatography on Pharmacia PD-10 Sephadex G-25

columns (Piscataway, N.J.). The affinity column was eluted again using the same procedure after standing at room temperature for several hours (up to 24 hours). Usually, this procedure was used rather than continuous elution because it provided better yield. Column fractions were monitored for protein by absorbance at 280 nm. For all other purposes, protein was determined by the method of Lowry et al[33] as modified by Peterson[34] using BSA as standard. Sodium dodecyi sulfate (SDS)-gel electrophoresis of column fractions or concentrated antibody was performed under the conditions of Laemmli[35] using a 5-16% linear gradient with a 5% stacking gel.

Titer, Affinity, and Specificity of Anti-Dihydropyridine Antibodies

A competitive [^{3}H]nitrendipine radioimmunoassay performed under equilibrium conditions as described previously[32] was used to determine the Specificity and affinity of the anti-dihydropyridine antibodies. Dextran-coated charcoal was used to separate free and bound [^{3}H]nitrendipine.[32] The competitive assay was performed using 20 pM [^{3}H]nitrendipine and various concentrations of potential inhibitor (10^{-12} to 10^{-4} M). A liter test performed prior to the competition assay identified the antibody dilution required to bind approximately 50% [^{3}H]nitrendipine in the absence of inhibitor. Average dissociation constants, antibody Specificity, and concentrations of specific antibodies in serum or affinity-purified preparations were calculated according to the methods of Müller.[36]

Immunoblot Staining of BSA-Azidopine

To prepare BSA-azidopine, 75 μl [^{3}H]azidopine (18.8 μM) in ethanol was mixed with 100 fig BSA (fraction V) in 250 μl distilled water. The mixture was irradiated with 254 nm UV light (Mineralite UVSL-25, Ultra-Violet Products, Inc., San Gabriel, Calif., and Spectroline ENF-24 lamps, Spectronic Corp., Westbury, N.Y.) for 2 minutes. Another 75- μl aliquot of [^{3}H]azidopine (18.8 μl) was then added, and the mixture was irradiated for 2.5 minutes. The mixture was evaporated to dryness in a Savant Speedvac and taken up in 100 μl water. Analysis of the counts of [^{3}H]azidopine in BSA after SDS-polyacrylamide gel electrophoresis showed that approximately 190 fmol [^{3}H]azidopine/μg BSA was incorporated. For immunoblot analysis, BSA and BSA-azidopine (40 μg each) were subjected to SDS-polyacrylamide gel electrophoresis using a 5-16% gradient gel with a 5% stacking gel,[35] and the proteins were electrophoretically transferred to nitrocellulose membranes.[37] The nitrocellulose blots were blocked with 5% nonfat dry milk in 0.9% Nad and 50 mM NaH_2PO_4 (pH 7.4) and then incubated with affinity-purified anti-dihydropyridine antibody (1:500 dilution in blocking solution) overnight in the presence or absence of 1 μM nitrendipine. The blots were then washed with 5% nonfat dry milk in 0.9% NaCl and 50 mM NaH_2PO_4 (pH 7.4) and incubated with affinity-purified peroxidase-conjugated rabbit anti-sheep IgG followed by washing and incubation with affinity-purified peroxidase-conjugated goat anti-rabbit IgG. After a final washing step, the blots were developed using 4-chloro-l-naphthol as the substrate.

Materials

[Methy-^{3}H]PN 200-110 (70-85 Ci/mmol) and [^{3}H]azidopine (45 Ci/mmol) were generously supplied by Amersham (Arlington Heights, Ill.). [^{3}H]Nitrendipine (88 Ci/mmol), [^{3}H]verapamil (85 Ci/mmol), [^{3}H]diltiazem (72 Ci/mmol), [^{3}H]azidopine (53 Ci/mmol), 1,4-dihydro-2,6-dimethyl-4-(2-isothiocyanatophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester, and [3-methy^{3}H]-1,4-dihydro-2,6-dimethy-4-(2-isothiocyanatophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (76.6 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). Nitrendipine, nisoldipine, and Bay K 8644 were generously supplied by Miles Laboratories (New Haven, Conn.). Nifedipine, verapamil, and diltiazem were generously supplied by Pfizer (New York, N.Y.), Knoll Pharmaceutical Co. (Whippany, N.J.), and Marion Laboratories (Kansas City, Mo.), respectively. Ryanodine was obtained from Penick (Lyndhurst, N.J.). Peroxidase-conjugated affinity-purified rabbit anti-sheep IgG secondary antibody was obtained from Bio-Rad Laboratories. Peroxidase-conjugated affinity-purified goat anti-rabbit IgG was obtained from Cooper Biomedical. Bovine serum albumin (fraction V), keyhole limpet hemocyanin, dextran (approximate molecular weight, 70,000), and l-ethyl-3-(3-dimethylaminopropyl)carbodiimide were obtained from Sigma Chemical Company (St. Louis, Mo.). Charcoal (Norif A) was obtained from Fisher. Dextran-coated charcoal was prepared by mixing 62.5 mg Dextran-70,625 mg Norit A charcoal, 100 ml 150 mM NaCI, and 10 mM Tris-HC1 (pH 7.2). Diethyl l,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate was obtained from Aldrich. All other reagents were of reagent grade quality.

Results

Synthesis of Dihydropyridine-Sepharose Affinity Matrix

Nifedipine-isothiocyanate was used to covalently incorporate nifedipine into aminohexyl-Sepharose beads as described in "Materials and Methods" and outlined in Figure 1. Incorporation of nifedipine-isothiocyanate was monitored by including [^{3}H]nifedipine-isothiocyanate in the reaction mixture. To prevent potential problems associated with ion exchange, free amine groups remaining after derivatization with nifedipine-isothiocyanate were blocked by reaction with acetic acid in the presence of l-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Nifedipine groups present in the final product ranged from 75-300 nmol/ml gel (quantity depended on the conditions used).

Affinity Purification of Anti-Dihydropyridine Antibodies

The nifedipine derivative of aminohexyl-Sepharose with amine groups blocked by acetylation was used to

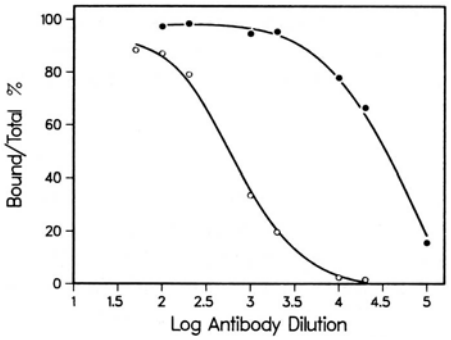

FIGURE 2. *Titer tests of serum and void of acetylated dihydropyridine-aminohexyl-Sepharose. [^{3}H]Nitrendipine-binding activity was determined for dilutions from 1:100 to 1:20,000 of hyperimmune sheep serum* (●) *and void from a column of acetylated dihydropyridine-aminohexyl-Sepharose* (○) *as described in "Materials and Methods." Tilers -were determined to be 37,000 for serum and 740 for column void.*

affinity purify anti-dihydropyridine antibodies. An affinity column was constructed using 5 ml affinity matrix. Figure 2 illustrates the ability of this column to absorb [^{3}H]nitrendipine-binding activity from serum. In this experiment, 50 ml sheep serum containing a high liter of anti-dihydropyridine antibody was pumped slowly over the affinity column several times. The original serum titer of 37,000 was reduced to a titer of 740 in the column void.

Elution of antibodies from affinity columns has often proven to be a difficult task requiring acidic or basic solutions[38] that partially denature the antibodies to elute them from the affinity matrix. Elution of active anti-dihydropyridine-specific antibodies was found to be possible using 50 mM diethylamine (pH 11.5) in 10% dioxane (data not shown). However, a more gentle method was suggested by the data of Loev et al39 in which the dihydropyridine derivative (diethyl 1, 4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate) was found to produce hypotension in dogs but produced hypotension with considerably lower potency than derivatives resembling those currently in clinical use with cyclic substituents in the 4 position. A competitive radioimmunoassay on hyperimmune sheep serum, performed as described in "Materials and Methods" and below, established that this compound competes with nitrendipine for dihydropyridine-binding sites on the antibody. However, the concentration (380 nM) required for half maximal inhibition of binding (IC_{50}) is more than 1,000 times greater than the IC_{50} for unlabelled nitrendipine, 0.34 nM (see Figure 3). This comparison suggested that this compound could be used to specifically elute anti-dihydropyridine antibodies but would be removable from eluted antibody by simple, rapid techniques. Elution with this compound was initially achieved in combination with 10% 1,4-dioxane. A typical purification run, in which antibody from 50 ml sheep serum was absorbed by a 5-ml affinity column, yielded 36 mg highly pure antibody when the column was eluted by this method. In subsequent experiments, elution was performed in the absence of dioxane. In Figure 4, an affinity column was saturated with anti-dihydropyridine antibody, washed, and then eluted with diethyl l,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate in the absence of dioxane. After elution of a peak of antibody, the column flow was stopped, the column was reequilibrated with elution buffer, and another peak of antibody was eluted.

Characterization of Affinity-Purified Anti-Dihydropyridine Antibodies

Affinity of the anti-dihydropyridine antibodies for [^{3}H]nitrendipine and the ability of various compounds to inhibit binding was determined using the competitive radioimmunoassay of Muller[36] as described previously.[32] The K_d for nitrendipine or the IC_{50} for other inhibitors was determined from the binding curve generated by adding unlabelled inhibitor at various concentrations to a 20-pM solution of [^{3}H]nitrendipine and

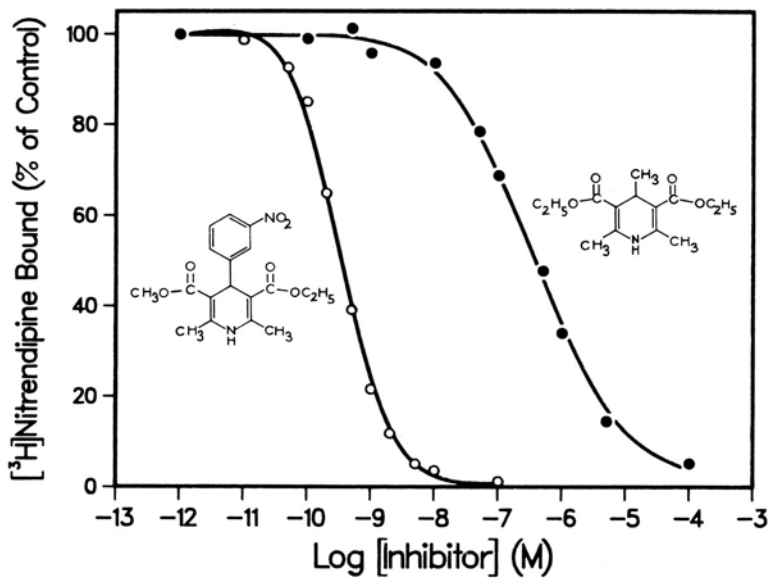

FIGURE 3. *Displacement of [^{3}H]nitrendipine from sheep serum anti-dihydropyridine by 1,4-diethyl-l,4-dihydro-2,4,6-trimethyt-3,5-pyridinedicarboxylate. Binding of [^{3}H]nitrendipine from 20-pM solution was measured in presence of increasing concentrations of unlabelled nitrendipine (10^{-12} to 10^{-7} M) (○) or diethyl 1,4-dihydro-2,4,6,-trimethyl-3,5-pyridinedicarboxylate (10^{-12} to 10^{-4} M) (●). IC_{50} for diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate was 380 nM compared with 0.34 nM for nitrendipine.*

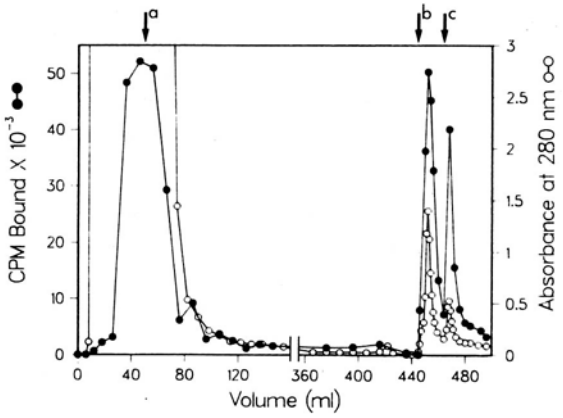

FIGURE 4. *Affinity chromatography of sheep anti-dihydropyridine antibodies. A 3.5-ml affinity column was prepared as described in "Materials and Methods." Approximately 50 ml hyperimmune sheep serum was pumped through the column once. Bulk of serum protein* (○) *was not retained by column, while [*^{3}*H]nitrendipine-bindmg activity* (●) *was absorbed until column became saturated. Column was then washed with 0.5 M Nad and 50 mM*NaH_2PO_4 *(pH 7.4) starting at arrow a. Beginning at arrow b,* 10^{-4} *M diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate was used to elute 1,4-dihydropyridine-specific antibodies. After first peak was eluted, column flow was stopped to allow column to reequilibrate with elution buffer (arrow c). Flow was then restored, resulting in elution of second antibody peak. Each fraction was sampled and assayed for [*^{3}*H]nitrendipine-binding activity* (●) *as described in "Materials and Methods." Protein was monitored by absorbance at 280 nm* (○).

antibody at the proper dilution to bind approximately 50% of label in the absence of inhibitor. The results of this analysis are shown in Figure 5 and Table 1 for antibodies eluted using diethyl l,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate with 10% dioxane and then subjected to gel nitration on Sephadex G-25. The apparent K_d for this preparation was found to be 0.1 nM (Figure 5a).

SDS-polyacrylamide gel electrophoresis was used to examine the purity of affinity-purified anti-dihydropyridine antibodies. Approximately 25 μg protein eluted by the harshest method employed (50 mM diethylamine (pH 11.5) in 10% dioxane) when subjected to electrophoresis on a 5-16% gradient gel under reducing conditions (Figure 5b) consisted of one heavy chain immunoglobulin band and one light chain band. Electrophoresis under nonreducing conditions (not shown) reveals a band of approximately 150,000 daltons, indicating that the purified antibody is of the IgG class.

The specificity of affinity-purified antibody for binding to various dihydropyridines and other calcium antagonists was also examined. Competitive radioimmunoassays in the presence of nifedipine, nisoldipine, Bay K 8644, verapamil, diltiazem, or ryanodine are summarized in Table 1. These established that only the dihydropyridine compounds compete for binding of [^{3}H]nitrendipine. The IC_{50}, for nifedipine, nisoldipine, and Bay K 8644 was 0.92, 6.8, and 0.80 nM, respectively. Like ryanodine, which is considered to be an inhibitor of sarcoplasmic reticulum Ca^{2+} channels,[40-41] verapamil and diltiazem, structurally unrelated Ca^{2+} channel blockers, produced little or no change in binding.

Immunoblot Characterization of Anti-Dihydropyridine Antibodies

Azidopine, an arylazide derivative of the dihydropyridine Ca^{2+} channel blockers,[42] was photocoupled to BSA, and the BSA-azidopine conjugate was used to test the ability of the affinity-purified antibodies to recognize 1,4-dihydropyridines covalently attached to protein. Control BSA and BSA-azidopine conjugate were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose paper. The nitrocellulose blots were blocked with 5% nonfat dry milk and then incubated with affinity-purified anti-dihydropyridine antibodies in the presence or absence of 1 μM nitrendipine. The blots were treated with peroxidase-conjugated rabbit anti-sheep IgG antibodies followed by peroxidase-conjugated goat anti-rabbit IgG antibodies. Development revealed that the anti-dihydropyridine antibodies reacted with the BSA-azidopine conjugate and not with unlabelled BSA (Figure 6). The reaction with the BSA-azidopine conjugate was blocked by the presence of 1 μM nitrendipine during the primary incubation. Therefore, the anti-dihydropyridine antibodies specifically detect azidopine covalently photocoupled to BSA.

Discussion

We have previously described the production in rabbits of sera-containing antibodies that resemble the

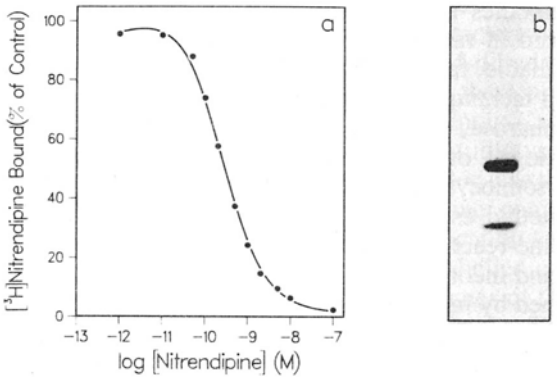

FIGURE 5. *Average dissociation constant and SDS-gel electrophoresis of affinity-purified anti-dihydropyridine antibody.* Panel a: *Average dissociaton constant of affinity-purified antibody preparation was determined using competitive radioimmunoassay of Muller*[33] *as described previously*[32] *and in "Materials and Methods." Antibody in this experiment was eluted using 1 MNaCl, 50 mM*NaH_2PO_4 *(pH 7.4), and 10-4 M diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate in 10% dioxane, which was subsequently removed by gel filtration on Sephadex G-25. Average antibody dissociation constant was determined to be 0.1 nM.* Panel b: *23 μg affinity-purified antibody eluted with 50 mM diethylamine and 0.5 M NaCl in 10% dioxane was subjected to SDS-gel electrophoresis on 5-16% gradient using 5% stacking gel under conditions of Laemmli.*[36]

Table 1. Inhibition of [^{3}H]Nitrendipine Binding to Affinity-Purified Antibodies by Various Ca^{2+} Channel Drugs and Related Compounds*

Compound	Percent inhibition at 10^{-7} M†	IC_{50} (nM)‡
Dihydropyridines		
Nitrendipine	98.0	0.23
Nisoldipine	88.0	6.80
Nifedipine	98.0	0.92
Bay K 8644	94.0	0.80
Ca^{2+} Blockers and antagonists		
Diltiazem	5.0	>1,000
Verapamil	11.0	>1,000
Ryanodine	<1.0	>1,000

*[^{3}H]Nitrendipine binding to and-dihydropyridine antibodies was performed using the dextran-coated charcoal assay as described in "Materials and Methods." Inhibition of [^{3}H]nitrendipine binding to the dihydropyridine-specific antibodies was measured by the addition of various unlabelled drugs or reagents (10^{-12} to 10^{-4} M) in the presence of 20 pM [^{3}H]nitrendipine.

†Inhibition of [^{3}H]nitrendipine binding at 10^{-7} M of unlabelled drug or reagent.

‡Half-maximal inhibition (IC_{50}) was determined from plot of percent [^{3}H]nitrendipine bound versus concentration of drug or reagent.

membrane receptor for the dihydropyridine Ca^{2+} channel blockers.[32] Since the antibodies mimic the membrane dihydropyridine receptor in their dihydropyridine-binding properties, they provide an ideal model for development of techniques, such as affinity purification, that may be applicable to the membrane receptor. In addition, affinity-purified antibodies are preferable for most applications. The production of antibodies in sheep similar to those previously produced in rabbits has made large quantities of serum available, facilitating development of affinity-purification techniques. An affinity matrix, dihydropyridine-Sepharose, was produced by coupling an affinity analogue of nifedipine, l,4-dihydro-2,6-dimethyl-4-(2-isothiocyanatophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester, to aminohexyl-Sepharose 4B via the amine-reactive isothiocyanate group. The amount of ligand incorporated into the affinity matrix was determined by including labelled compound in the reaction mixture. The resulting Sepharose-derivative retains the moieties essential for potent pharmacologic activity, i.e., the 1,4-dihydropyridine ring with a bulky substituent in the 4 position.[39,43] To prevent the matrix from acting as an ion exchanger, residual primary amine groups left after coupling of nifedipine-isothiocyanate were blocked by reaction with acetic acid using l-ethyl-3-(3-dimethylaminopropyl) carbodiimide, a water-soluble carbodiimide, as catalyst. These procedures yielded an affinity matrix capable of specifically binding anti-dihydropyridine activity from serum.

Elution of high-affinity antibodies from affinity columns is often difficult[38] and was for anti-dihydropyridine antibodies. Traditional methods of elution involve extremes of pH, use of protein denaturants such as urea or guanidine-HCl, or use of chaotropic salts.[38] However, all these methods are potentially denaturing. More gentle methods of dissociation of antigen-antibody complexes that have been successfully employed include elution with distilled water[44] or free hapten in high concentration (e.g., see Lowe and Dean[38]). It has sometimes proven helpful to include organic solvents such as diethyl ether,[45] ethylene glycol,[46] or 1,4-dioxane[46,47] in the elution medium.

We have successfully eluted anti-dihydropyridine antibodies using a basic solution in 10% dioxane. However, we were interested in developing a more gentle method using neutral conditions that might also

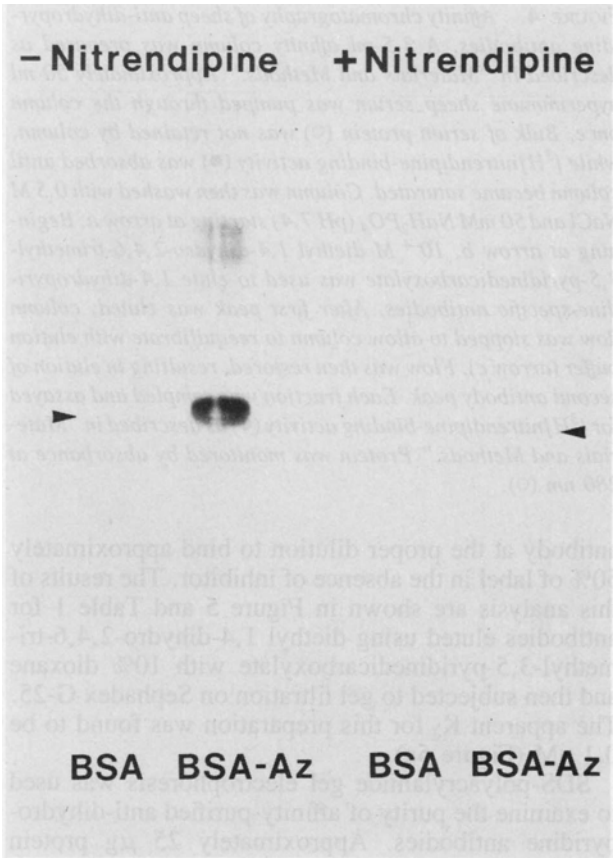

FIGURE 6. *Indirect immunoperoxidase staining of dihydropyridine-labelled bovine serum albumin by affinity-purified anti-dihydropyridine antibodies. Binding of affinity-purified anti-dihydropyridine antibodies to bovine serum albumin conjugates of azidopine was tested by indirect immunostaining as detailed in "Materials and Methods." Control bovine serum albumin (BSA) and bovine serum albumin-azidopine conjugate (BSA-Az) (40 µg each) were subjected to SDS-polyacrylamide gel electrophoresis[36] and transferred to nitrocellulose paper.[37] Nitrocellulose blots were incubated overnight with affinity-purified anti-dihydropyridine antibodies (1:500 dilution) in absence of nitrendipine, (–)nitrendipine, or presence of 1 µM nitrendipine, (+)nitrendipine. Following 1-hour incubations with peroxidase-conjugated affinity-purified rabbit anti-sheep IgG antibody and peroxidase-conjugated affinity-purified goat anti-rabbit IgG antibody, blots were developed using 4-chloro-1-naphthol as substrate. Arrow indicates position of BSA.*

be applicable to purification of membrane receptors for the 1,4-dihydropyridine Ca^{2+} channel blockers. While the use of free ligand in high concentration often allows elution under mild conditions,[38] the ligand may in some cases be difficult to obtain or may be of limited solubility. In addition, it may be difficult to remove a very high affinity ligand from antibodies or receptors following elution.

For these reasons, we developed a method to elute anti-dihydropyridine antibodies using a low-affinity ligand, diethyl l,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate, in combination with 10% dioxane at neutral pH. This method permits large quantities of highly pure antibody to be rapidly isolated from serum without exposing the antibody to extremes of pH. Subsequently, it was found that elution could be performed using diethyl l,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate in the absence of dioxane, allowing high-affinity antibodies to be isolated under the mildest possible conditions. In addition, since the eluting ligand is of relatively low affinity (IC_{50} for [^{3}H]nitrendipine binding = 380 nM), it is easily removed by rapid gel nitration chromatography.

Potential uses of antiligand antibodies include identification and immunoaffinity purification of endogenous analogues of high-affinity drugs such as the endogenous ligands that have been found for the cardiac glycoside receptor[28] and the apamin binding Ca^{2+} dependent K^+ channel.[27] In addition, antiligand antibodies may be used to inoculate animals to produce antiidiotypic antibodies that bind to the receptor for the original ligand and may mimic the physiologic effects of the ligand.[19,26]

To be useful for these applications, it is necessary for the antiligand antibodies to mimic the binding characteristics of the membrane receptor. Therefore, the affinity and specificity of the purified antibodies was examined to establish that the methods used permit retention of binding characteristics similar to those of the membrane receptor for the 1,4-dihydropyridine Ca^{2+} channel blockers. The average dissociation constant of the purified antibody preparation for [^{3}H]nitrendipine as determined by competitive radioimmunoassay was 0.1 nM (Figure 5). This is similar to the nitrendipine-binding affinity of the membrane receptor for 1,4-dihydropyridine Ca^{2+} channel blockers.[9-13] The competitive radioimmunoassay was also used to examine specificity of the purified antibodies. All 1,4-dihydropyridine Ca^{2+} channel blockers tested, as well as Bay K 8644 (a Ca^{2+} channel activator[12]), potently inhibited binding of [^{3}H]nitrendipine to the antibodies. The ability of diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate to elute antibodies from the affinity column indicates that this compound is also probably capable of competing for binding sites on purified antibodies. Diethyl l,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate is known to have some hypotensive activity[39] and is a relatively weak inhibitor of binding of [^{3}H]PN 200-110 to the membrane receptor for 1,4-dihydropyridine Ca^{2+} channel blockers (data not shown). Therefore, the affinity-purified anti-dihydropyridine antibodies and the receptor have similar binding properties for dihydropyridines.

The Ca^{2+} channel blockers verapamil and diltiazem, as well as ryanodine (an activator of sarcoplasmic reticulum Ca^{2+} channels), do not significantly alter binding of pHjnitrendipine, which indicates that they do not compete for dihydropyridine-binding sites on the antibodies. Although verapamil inhibits and diltiazem stimulates binding of 1,4-dihydropyridine Ca^{2+} channel blockers to membranes, these effects are not via competition for dihydropyridine-binding sites.[8,13] Therefore, both the affinity and specificity of affinity-purified anti-dihydropyridine antibodies are in agreement with those of the membrane dihydropyridine-binding sites. Further strengthening the parallels between binding properties of the antibodies and the membrane receptor for the 1,4-dihydropyridine Ca^{2+} channel blockers, [^{3}H]PN 200-110-binding activity from rabbit transverse tubular membrane preparations has been shown to be specifically absorbed by the dihydropyridine-Sepharose affinity columns (not shown).

Another potential use of affinity-purified anti-dihydropyridine antibodies is identification and purification of dihydropyridine receptors labelled covalently with affinity or photoafflnity analogues of the 1,4-dihydropyridines. We have shown that the antibodies recognize protein conjugates of azidopine (Figure 6) and nifedipine-isothiocyanate (not shown). Therefore, these antibodies may be useful for detection and purification of receptors or receptor peptides labelled with azidopine or nifedipine-isothiocyanate.

In summary, methods have been developed for production and purification under mild conditions of large quantities of highly pure anti-dihydropyridine antibodies. The antibodies retain binding characteristics similar to the membrane receptor for the 1,4-dihydropyridine Ca^{2+} channel blockers and should, therefore, be useful in identification of possible endogenous Ca^{2+} channel ligands and for production of antiidiotypic antibodies against the membrane receptor. Because the antibodies recognize protein-dihydropyridine conjugates, they should also be useful for identification and purification of dihydropyridine receptors labelled covalently with affinity or photoafflnity analogues of the 1,4-dihydropyridine Ca^{2+} channel blockers. The affinity column and mild elution methods developed for purification of the antibodies may also prove applicable to purification in active form of the membrane receptor for the 1,4-dihydropyridine Ca^{2+} channel blockers.

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