BIOORGANIC CHEMISTRY 17, 141–158 (1989)

Molecular Tools of Calcium Channel Studying: Synthesis and Evaluation of Biological Activity of Novel Dihydropyridine Calcium Entry Blockers

NIKOLAI M. SOLDATOV

Institute of Biomedical Technology, USSR Ministry of Public Health, 6, Schukinskaya str., 123436 Moscow, USSR

Received May 25, 1988

A series of novel dihydropyridine (DHP) calcium entry blockers has been synthesized and evaluated for their biological activity in an attempt to develop specific pharmacological tools of calcium channel studying. The series includes (i) radioactively labeled ethoxycarbonyl derivative of nicardipine; (ii) [^{125}I]iodo DHP congener; (iii) two radioactive photoactivable DHP derivatives; (iv) DHP-Sepharose affinity resin; and (v) bovine serum albumin conjugate suitable for the production of anti-DHP antibodies. All the compounds have been generated from a single key 3-hydroxyethoxycarbonyl DHP derivative. The dihydropyridine compounds obtained exhibit a high level of biological activity in radioligand binding studies with rabbit skeletal muscle membranes. These properties of novel DHP derivatives suggest their potential utility as valuable pharmacological tools in studies of dihydropyridine receptor, a putative calcium channel.

INTRODUCTION

Calcium antagonists are increasingly used to explore the molecular nature of calcium ions' translocation through biological membranes. Ranked among the most potent calcium antagonists, 4-aryl-1,4-dihydropyridines (DHP)[1] have proven to have remarkable pharmacological properties of calcium entry blockers (*1*). Direct binding studies revealed that DHPs interact stereospecifically with a single class of saturable binding sites in plasma membranes of nerve and muscle cells with affinity constants (K_d) in the range 10^{-10}–10^{-9} M (*2–4*). These binding sites are related to long-lasting, or so-called type L, calcium channels, and in a variety of patch-clamp experiments DHP calcium antagonists were shown to decrease the probability of the opening of these channels (*5–7*). This spectrum of properties underlies the wide application of DHP derivatives as vasodilators in the treatment of various cardiovascular disorders (*8*) and has proven DHP to be a valuable tool for the study of voltage-dependent calcium channels.

While starting the investigation of the molecular properties of DHP-sensitive calcium channels, we found it necessary to prepare a number of specific DHP

[1] Abbreviations used: Chapso, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; DHP, dihydropyridine; BSA, bovine serum albumin.

0045-2068/89 $3.00

derivatives which could provide the detection, labeling, and biospecific isolation of the DHP receptor. The present study was undertaken in an attempt to evaluate a series of novel DHP derivatives suitable for receptor isolation and investigation including both radioactively labeled and photoactivable compounds, anti-DHP antibodies, and affinity resin containing DHP molecules covalently attached to Sepharose beads. This has been achieved starting from a single key compound, the 3-hydroxyethoxycarbonyl derivative of DHP.

EXPERIMENTAL PROCEDURES

General

All reactions carried out at room temperature were at or near 20°C. Solvents were dried or distilled before use. Unless otherwise indicated, all reactions were carried out under an inert atmosphere of argon with stirring. During workup, solvents were removed with a rotary evaporator unless otherwise stated.

Proton magnetic resonance spectra were recorded on either a Varian SC-300 NMR spectrometer (300 MHz) or a Brüker WM-500 instrument (500 MHz) using $CDCl_3$ as solvent. Chemical shifts are reported in δ units, parts per million (ppm) downfield from Me_4Si. Mass spectra were obtained on a Varian MAT CH-5 or Mat 44c analyzers. Melting points are uncorrected.

Chemicals. All analytical TLC work was done with Kieselgel 60 F_{254} plates (E. Merck, 0.20-mm layer). Preparative TLC was carried out on DC-Alufolien Kieselgel 60 plates. Chromatographic purifications were carried out with Kieselgel 100 as the solid phase (70–230 mesh), from E. Merck Laboratories. The following starting materials and special reagents were purchased from the sources listed and were used without additional purification unless otherwise stated: diketen (Ferak), 3-aminobenzoic acid, anthranilic acid, ethyl chloroformate, methane sulfonyl chloride, and polyethylene glycol 6000 (Fluka AG); sodium borohydride (BDH Chemicals Ltd); 4-dimethylaminopyridine and succinic anhydride (Aldrich Fine Chemicals); 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and triethylamine (Pierce Eurochemie B.V.); AH-Sepharose 4B (Pharmacia Fine Chemicals); and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (Protein Research Foundation). The following biochemicals were used: D-*cis*-diltiazem (Gödecke); bovine serum albumin (fraction V) and phenylmethylsulfonyl fluoride (Sigma Chemical Co.); goat anti-rabbit globulin (Progressive Labs); Freund's adjuvant, complete and incomplete (Calbiochem-Behring); and GF/C glass fiber filters (Whatman Ltd). Other chemicals were of analytical grade.

The following radioisotopes were used: sodium boro[^{3}H]hydride at 8 Ci/mmol and sodium [^{125}I]iodide, carrier free, were from Amersham, England; 2-amino-[5-^{3}H]benzoic acid at 29 Ci/mmol and 4-amino-[3,5-$^3H_2$]benzoic acid at 59 Ci/mmol were prepared from 5-iodoanthranilic acid and 3,5-diiodo-4-aminobenzoic acid, respectively, by catalytic hydrogenation under an atmosphere of tritium gas (Institute of Molecular Genetics, USSR Academy of Sciences, Moscow).

Radioactivity was analyzed using Beckman LS 9800 or Intertechnique liquid

scintillation spectrometers. Radioactive samples were dissolved in Beckman Ready-Solv NA scintillation mixture for counting. Biological samples were analyzed in scintillation cocktail containing 4 g of Scintimix-2 (Koch-Light) and 160 ml of Bio-Solv 3 (Beckman) per 1 liter of toluene solution, or in Supersolve X (Koch-Light). Patterns of TLC separations of radioactive mixtures were analyzed with Berthold LB 2832 automatic linear analyzer.

Synthetic Procedures

Hantzsch synthesis. A mixture of 15.5 g (110 mmol) of ethyl 3-aminocrotonate, 16.5 g (100 mmol) of 2-chloroethyl acetoacetate, and 18.1 g (120 mmol) of *m*-nitrobenzaldehyde dissolved in 28 ml of isopropanol was refluxed for 4 h. Then the mixture was cooled and concentrated under reduced pressure. The residue was recrystallized by dissolution in 30 ml of boiling EtOAc. This afforded about 18 g of a mixture that consisted essentially of compounds **1** and **2**. The yellow crystals obtained were washed several times with cold diethyl ether, dissolved in a small volume of EtOAc, and adsorbed onto a chromatography column with silica gel packed in EtOAc : hexane (1 : 2). The column was eluted first with EtOAc : hexane (1 : 2) to afford fraction of chloride 2 and then with EtOAc : hexane (1 : 1) to release hydroxy compound **1.**

2,6-Dimethyl-3-(2-chloroethoxycarbonyl)-5-ethoxycarbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine (**2**). This compound was recrystallized from EtOAc to yield 12.7 g (31%); mp 146–148°C (dec.); TLC R_f 0.65 (EtOAc : hexane, 2 : 1); mass spectrum, *m/e* 408, 410 (M^+); ^1H NMR δ 1.23 (t, 3H, $-COOCH_2CH_3$), 2.37 (s) and 2.39 (s) (6H, $-CH_3$), 3.64 (t, 2H, $-CH_2Cl$), 4.10 (m, 2H, $-CH_2CH_3$), 4.30 (m, 4H, $-CH_2CH_2-$), 5.12 (s, 1H, C(4)–H), 5.88 (s, 1H, N–H), 7.39 (t, 1H, Ph–H(5)), 7.68 (d, 1H, Ph–H(4)), 8.01 (d, 1H, Ph–H(6)), and 8.14 (s, 1H, Ph–H(2)).

2,6-Dimethyl-3-(2-hydroxyethoxycarbonyl)-5-ethoxycarbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine (***1***). This compound was obtained from the column fraction as a thick oil in 4.1% overall yield. Additional purification of the titled compound was provided by the reversible silylation of **1** with *N*-trimethylsilyldiethylamine at 1 : 4 molar ratio in dry acetone at room temperature. After the solution was stirred for 24 h, TLC analysis ($CHCl_3$: EtOAc : hexane, 1 : 1 : 1) indicated clean formation of trimethylsilyl derivative of **1** (R_f = 0.46). The reaction mixture was concentrated to dryness, and the derivatized alcohol was isolated by column chromatography on silica gel equilibrated with $CHCl_3$: EtOAc : hexane (5 : 5 : 7). Pooled fractions were evaporated *in vacuo,* the residue was dissolved in methanol and made acidic by addition of HCl solution in methanol. After 30 min at room temperature, the solvent was removed *in vacuo.* The yellow oily product formed crystals within 2–3 days to give **1** with ca. 90% yield. Mass spectrum, *m/e* 390 (M^+); TLC R_f 0.26 (EtOAc : hexane, 2 : 1); ^1H NMR δ 1.25 (t, 3H, $-COOCH_2CH_3$), 2.36 (s) and 2.38 (s) (6H, $-CH_3$), 3.80 (m, 2H, $-CH_2CH_3$), 4.20 (m, 4H, $-CH_2CH_2-$), 5.16 (s, 1H, C(4)–H), 6.08 (s, 1H, N–H), 7.43 (t, 1H, Ph–H(5)), 7.74 (d, 1H, Ph–H(4)), 8.08 (d, 1H, Ph–H(6)), and 8.22 (s, 1H, Ph–H(2)).

Compound **1** formed the corresponding acetate when treated with an excess of acetic anhydride in absolute pyridine overnight at room temperature. The

resulting 2,6-dimethyl-3-(2-acetoxyethoxycarbonyl)-5-ethoxycarbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine was isolated by TLC on a silica gel plate in EtOAc: hexane (2:1), R_f 0.58. Mass spectrum, *m/e* 432 (M^+).

2,6-Dimethyl-3-(2-bromoethoxycarbonyl)-5-ethoxycarbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine (***10***). A solution of 975 mg (2.5 mmol) of **1** in 4 ml of absolute pyridine was treated with 0.5 ml of methane sulfonyl chloride at 0°C for 2 h under argon. Then 5 ml of chloroform was added and the organic layer was washed with 3 × 0.5-ml portions of 3% $NaHCO_3$, 2 × 0.5 ml of saturated NaCl solution and dried over sodium sulfate. The solvent was evaporated *in vacuo,* and the oily residue was dissolved in 4 ml of absolute acetone and treated with the solution of 1.2 g of anhydrous LiBr in 6 ml of absolute acetone at room temperature. After 24 h, TLC analysis (EtOAc: hexane, 2:1) indicated the complete conversion of mesylate (R_f 0.5) to bromide **10** (R_f 0.68). The solvent was removed on a rotary evaporator to give ca. 1.0 g of the crude material. This was chromatographed on silica gel column (2.5 × 35 cm) with EtOAc: hexane (2:3) as eluent. After the solvent was evaporated *in vacuo*, the residue was recrystallized from methanol to give 855 mg (75.5%) of compound **10** as bright yellow crystals; mp 153–155°C (dec.); mass spectrum, *m/e* 452, 454 (M^+); ^{1}H NMR δ 1.25 (t, 3H, $-COOCH_2CH_3$), 2.37 (s) and 2.39 (s) (6H, $-CH_3$), 3.46 (t, 2H, $-CH_2Br$), 4.12 (m, 2H, $-CH_2CH_3$), 4.37 (m, 4H, $-CH_2CH_2-$), 5.13 (s, 1H, C(4)–H), 5.88 (s, 1H, N–H), 7.39 (t, 1H, Ph–H(5)), 7.68 (d, 1H, Ph–H(4)), 8.01 (d, 1H, Ph–H(6)), and 8.14 (s, 1H, Ph–H(2)).

2,6-Dimethyl-3-[2-(N-benzyl-N-methylamino)ethoxycarbonyl]-5-ethoxycarbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine (***3b***). To a solution of 0.22 mmol (100 mg) of compound **10** in 0.5 ml of absolute toluene 59.6 μl (0.46 mmol) of benzylmethylamine was added and heated for 2 h at reflux. After solvent removal, the crude material was recrystallized from chloroform to give 89 mg (82% yield) of compound **3b;** mp 168–170°C (dec.); TLC R_f 0.26 (EtOAc: hexane, 3:2); mass spectrum, *m/e* 493 (M^+); ^{1}H NMR δ 1.22 (t, 3H, $-COOCH_2CH_3$), 2.38 (s) and 2.42 (s) (6H, $-CH_3$), 2.58 (s, 3H, $N-CH_3$), 3.20 (s, 2H, $-CH_2Ph$), 4.10 (m, 4H, $-CH_2CH_2-$), 4.60 (m, 2H, $-N-CH_2-$), 5.06 (s, 1H, C(4)–H), 6.84 (s, 1H, N–H), 7.32 (t, 1H, Bz–H(4)), 7.38 (3 t, 3H, Bz–H(3, 5) and Ph–H(5)), 7.53 (2 d, 2H, Bz–H(2,6)), 7.59 (d, 1H, Ph–H(4)), 7.95 (d, 1H, Ph–H(6)), and 8.05 (s, 1H, Ph–H(2)).

N-[^{3}H]benzyl-N-methylamine. Sodium boro[^{3}H]hydride (12.5 μmol) was dissolved at 0°C in 60 μl of 65% aqueous ethanol and mixed with a solution of 12.5 μmol of *N*-benzylidenemethylamine in 15 μl of ethanol. After being cooled in ice for 5 min, a reaction mixture was acidified cautiously with a solution of HCl in methanol to pH 3–4, neutralized with a solution of NH_3 in MeOH, and diluted with 300 μl of water. The radioactive product was extracted into chloroform and organic phase was dried (Na_2SO_4), filtered, and evaporated in a stream of argon to give the titled product in ca. 30% yield. TLC R_f 0.16 ($CHCl_3$: MeOH, 3:2); radiochemical purity, 60–70%.

2,6-Dimethyl-3-[2-(N-[^{3}H]benzyl-N-methylamino)ethoxycarbonyl]-5-ethoxycarbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine (***3a***). Compound **10** (1.1 μmol) dissolved in 15 μl of toluene was heated at 110°C for 1.5 h with *N*-[^{3}H]benzyl-*N*-methylamine in a tightly closed 0.3-ml Reacti-vial. The solvent was re-

moved on a rotary evaporator, the residue was taken up in 0.1 ml of chloroform and separated by TLC on a silica gel plate developed with EtOAc : hexane (2 : 1). Nonradioactive analog **3b** was used as a standard. The titled radioactive product was eluted with chloroform from zone with R_f 0.31. TLC showed that radiochemical purity of the target compound was ca. 90%. **3a** was stored as 50 μM solution in methanol at −35°C.

2,6-Dimethyl-3-(2-iodoethoxycarbonyl)-5-ethoxycarbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine (***4b***). This compound was prepared in the same manner as bromide **10** by treating the mesylate of compound **1** with a sevenfold molar excess of NaI in absolute acetone for 17 h at room temperature. The reaction mixture was diluted with water and extracted with chloroform. The extract was washed with 3% $NaHCO_3$ and saturated NaCl, dried (Na_2SO_4), and concentrated *in vacuo*. The residue was purified by silica gel chromatography developed with EtOAc : hexane (2 : 3) (R_f 0.63) to give yellow crystal solid with an overall yield of 75%; mp 149–151°C (dec.); mass spectrum, *m/e* 500 (M^+); ^1H NMR δ 1.26 (t, 3H, $-COOCH_2CH_3$), 2.39 (s) and 2.42 (s) (6H, $-CH_3$), 3.27 (t, 2H, $-CH_2I$), 4.12 (m, 2H, $-CH_2CH_3$), 4.33 (m, 4H, $-CH_2CH_2-$), 5.16 (s, 1H, C(4)–H), 5.98 (s, 1H, N–H), 7.42 (t, 1H, Ph–H(5)), 7.72 (d, 1H, Ph–H(4)), 8.05 (d, 1H, Ph–H(6)), and 8.19 (s, 1H, Ph–H(2)).

2,6-Dimethyl-3-(2-[^{125}I]iodoethoxycarbonyl)-5-ethoxycarbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine (***4a***). $Na^{125}I$ (0.32 nmol, 0.6 mCi) was freeze-dried from aqueous solution in an 0.8-ml Reacti-vial and mixed with 50 μl of a solution of 11 μmol of mesylate of compound **1** in absolute acetone. After standing at 25°C for 17 h, the reaction mixture was separated by TLC on silica gel plate developed in EtOAc : hexane (7 : 5) with **4b** as a standard. A radioactive spot relative to the titled compound was excised and extracted with chloroform to afford ^{125}I-labeled dihydropyridine **4a** with 55% yield and ca. 90% radiochemical purity.

2,6-Dimethyl-3-[2-(2-azidobenzoyl)ethoxycarbonyl]-5-ethoxycarbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine (***5b***). To a solution of 411 mg (3 mmol) of *o*-azidobenzoic acid in 105 ml of absolute benzene containing 431 μl (3.1 mmol) of triethylamine was added 287 μl (3 mmol) of ethyl chloroformate. After stirring at room temperature for 7 h, a solution of 100 mg (0.256 mmol) of compound **1** in a mixture of 30 ml of benzene and 3 ml of triethylamine was added. The reaction mixture was incubated for 10 days in the dark and then concentrated *in vacuo* under dim light and the titled compound was isolated in 55% yield from the residue by silica gel column chromatography with EtOAc : hexane (3 : 5) as eluent. TLC R_f 0.40 (EtOAc : hexane, 1 : 1); mp 48–50°C; mass spectrum, *m/e* 535 (M^+); ^1H NMR δ 1.17 (t, 3H, $-COOCH_2CH_3$), 2.35 (s) and 2.39 (s) (6H, $-CH_3$), 4.0 (m, 2H, $-CH_2CH_3$), 4.45 (m, 4H, $-CH_2CH_2-$), 5.12 (s, 1H, C(4)–H), 5.73 (s, 1H, N–H), 7.15 (t, 1H, N_3Ph–H), 7.22 (t, 1H, N_3Ph–H), 7.54 (t, 1H, Ph–H(5)), 7.60 (d, 1H, N_3Ph–H), 7.65 (d, 1H, N_3Ph–H), 7.75 (d, 1H, Ph–H(4)), 7.87 (d, 1H, Ph–H(6)), and 8.09 (s, 1H, Ph–H(2)).

2,6-Dimethyl-3-[2-(4-azidobenzoyl)ethoxycarbonyl]-5-ethoxycarbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine (***6b***). This compound was prepared in the same manner as **5b** starting from *p*-azidobenzoic acid and compound **1**. The target derivative was isolated by silica gel column chromatography using EtOAc : hex-

ane (1 : 1) as eluent (60% yield); mp 49–51°C: TLC R_f 0.6 (EtOAc : hexane, 1 : 1); mass spectrum, *m/e* 535 (M^+); ^1H NMR δ 1.20 (t, 3H, $-COOCH_2CH_3$), 2.36 (s) and 2.39 (s) (6H, $-CH_3$), 4.09 (m, 2H, $-CH_2CH_3$), 4.46 (m, 4H, $-CH_2CH_2-$), 5.10 (s, 1H, C(4)–H), 6.00 (s, 1H, N–H), 7.05 (d, 2H, N_3Ph–H), 7.24 (t, 1H, Ph–H(5)), 7.61 (d, 1H, Ph–H(4)), 7.89 (d, 1H, Ph–H(6)), 7.95 (d, 2H, N_3Ph–H), and 8.01 (s, 1H, Ph–H(2)).

2-Azido-[5-^3H]benzoic acid. An ice-cold solution of 2 μmol of 2-amino-[5-^{3}H]benzoic acid in 20 μl of 1% NaOH was treated under dim light with 0.4 mg of $NaNO_2$ and 2 μl of concentrated HCl. After stirring at 4°C for 30 min, 0.5 mg of NaN_3 was added, and then the reaction mixture was diluted with 1 ml of ice-cold water and extracted with chloroform (4 × 0.2 ml). The combined extract was dried by flow through a minicolumn with Na_2SO_4 and concentrated in a stream of argon. The titled product was obtained with ca. 30% yield and 48% radiochemical purity as determined by TLC in $CHCl_3$: MeOH (4 : 1), R_f 0.26.

4-Azido-[3,5-$^3H_2$]benzoic acid. This was prepared with 60% yield and ca. 50% radiochemical purity in a fashion similar to that of above compound starting from 4-amino-[3,5-$^3H_2$]benzoic acid. TLC R_f 0.56 ($CHCl_3$: MeOH, 9 : 1.5).

2,6-Dimethyl-3-[2-(2-azido-[5-^3H]benzoyl)ethoxycarbonyl]-5-ethoxycarbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine **(5a).** Ethyl chloroformate (1.2 μmol) in 3 μl of benzene was mixed with a solution of 1.2 μmol of 2-azido-[5-^{3}H]benzoic acid and 1.3 μmol of triethylamine in 40 μl of benzene. After incubation at room temperature for 5 h, a solution of 0.035 mg (0.09 μmol) of compound **1** and 1.2 μl of triethylamine in 12 μl of benzene was added. The mixture was left for 12–14 days at room temperature in dark. The titled product was isolated in 47% yield by TLC on silica gel plate developed with EtOAc : hexane (3 : 5) from a radioactive spot (R_f 0.4) relative to nonlabeled analog **5b** used as a reference. The molar radioactivity of **5a** was estimated to be ca. 27 Ci/mmol at 95% radiochemical purity. The compound obtained was dissolved in methanol and stored at −35°C.

2,6-Dimethyl-3-[2-(4-azido-[3,5-$^3H_2$]benzoyl)ethoxycarbonyl]-5-ethoxy-carbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine **(6a).** This compound was prepared in the same manner starting from 4-azido-[3,5-$^3H_2$]benzoic acid and compound **1** with 42% yield. Molar radioactivity was found to be 58 Ci/mmol at 92% radiochemical purity.

2,6-Dimethyl-3-(2-hemisuccinyloxyethoxycarbonyl)-5-ethoxycarbonyl-4-(3-nitrophenyl)-1,4-dihydropyridine **(7).** Succinic anhydride (1.0 g, 10 mmol) was added to a solution of 390 mg (1 mmol) of compound **1** in 50 ml of absolute pyridine containing 12.7 mg (0.1 mmol) of 4-dimethylaminopyridine. The reaction mixture was incubated in the dark at 25°C for 7 days. After the solvent was evaporated *in vacuo,* the residue was dissolved in 50 ml of chloroform, washed with water (3 × 20 ml) and the organic phase was dried (Na_2SO_4). The solvent was removed on a rotary evaporator and the residue was applied to a silica gel column (2.5 × 40 cm). The column was washed with 0.2 liter of EtOAc and then eluted subsequently with 0.1 liter of EtOAc : acetone (1 : 2) and with 0.5 liter of acetone. This afforded 372 mg (76% yield) of DHP hemisuccinate **7,** TLC R_f 0.35 (EtOAc : acetone, 1 : 2); mp 122–125°C (dec.); mass spectrum, *m/e* 490 (M^+); ^1H NMR δ 1.24 (t, 3H, $-COOCH_2CH_3$), 2.34 (s) and 2.35 (s) (6H, $-CH_3$), 2.67 (m, 4H,

$-CH_2-CH_2-$), 4.29 (m, 6H, $-CH_2-$), 5.09 (s, 1H, C(4)–H), 5.93 (s, 1H, N–H), 7.39 (t, 1H, Ph–H(5)), 7.66 (d, 1H, Ph–H(4)), 8.00 (d, 1H, Ph–H(6)), and 8.13 (s, 1H, Ph–H(2)).

Preparation of DHP-Sepharose 4B (**8**). Method a: AH-Sepharose 4B (5 g, ca. 40 μmol of free amino groups/ml gel) was resuspended in 200 ml of water, washed on a coarse disk sintered-glass funnel subsequently with 1 liter of 0.5 M NaCl, 250 ml of water, and equilibrated in 50% dioxane. The resin was resuspended in a mixture of 15 ml of dioxane and 10 ml of water (pH 5.0). A solution of 98 mg (0.2 mmol) of DHP hemisuccinate **7** in 2 ml of dioxane was added and the gel suspension was treated with 1 ml of aqueous solution of 1.15 g (6 mmol) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride at 20°C under continuous agitation in a rotating flask for 24 h at pH maintained at pH 5.0. Resin was filtered through a glass funnel with slight suction and washed subsequently with 200 ml of 50% aqueous dioxane, 300 ml of water, 100 ml of 5 mM HCl, 500 ml of water, 100 ml of 50% ethanol, 50 ml of 90% ethanol, and finally with 200 ml of water. Free amino groups of the resin were blocked by 17-h incubation in 0.2 M acetic acid. The affinity resin was then washed with 20 mM Tris · HCl buffer (pH 7.4) and stored in the same medium until further use.

Method b: 30 ml of prewashed AH-Sepharose 4B equilibrated in water was mixed with a solution of 193 mg (0.77 mmol) of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline in 12 ml of ethanol. Then a solution of 378 mg (0.77 mmol) of DHP hemisuccinate **7** in 13 ml of ethanol was added. The entire incubation was stirred at 18°C for 24 h. The reaction was stopped by filtering the gel suspension through a coarse disk sintered-glass funnel and the resin was washed repeatedly with 90% ethanol until the flow-through fractions revealed no hemissucinate **7**. Then the resin was washed subsequently with 1 liter of water and incubated overnight in 200 ml of 0.1 M acetic acid. The affinity resin was finally washed with 1 liter of 0.5 M NaCl and stored in 0.5 M NaCl containing 0.02% NaN_3 and 20 mM Tris · HCl (pH 7.4) until further use. The amount of DHP covalently linked to the resin by methods a and b was estimated to be 2–10 μmol/ml packed gel.

Coupling of DHP hemisuccinate 7 to bovine serum albumin. Bovine serum albumin (18 mg) dissolved in 1 ml of water was mixed with a solution of 17.5 mg (35.7 μmol) of DHP hemisuccinate **7** in 0.75 ml of dioxane. A solution of 19.8 mg (0.1 mmol) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride was then added. After stirring for 8 h at room temperature and pH maintained at pH 5.5, the reaction mixture was dialyzed against 0.5 liter of dioxane : water (1 : 4) for 12 h and against 0.5 liter of water for the next 12 h. The resulting insoluble conjugate **9** was freeze-dried and stored at −35°C.

Biochemical Procedures

Rabbit skeletal muscle sarcoplasmic reticulum and transverse tubule membranes were isolated according to Rosemblatt *et al.* (*9*) in the presence of 1 mM phenylmethane sulfonyl fluoride, 1 mM *o*-phenanthroline, 1 mM iodoacetamide, and 0.1 μM pepstatin A to minimize the proteolytic degradation.

Preparation of solubilized receptor. Transverse tubule membranes were solubi-

lized at 6 mg/ml protein concentration with 16 mM 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (Chapso) containing 5% glycerol and 20 mM Tris · HCl, pH 7.4. After centrifugation for 30 min at 150,000*g*, the supernatant was diluted 10-fold with 1.6 mM Chapso in the same buffer containing 0.01% dimiristoylphosphatidylcholine. This detergent extract was used for the determination of binding of the DHP receptor to affinity resin **8.** Solubilized receptor was measured by the polyethylene glycol precipitation method essentially as described by Glossmann and Ferry (*10*) with 2,6-dimethyl-3-methoxycarbonyl-5-([2,3-$^3H_2$]-*n*-propoxycarbonyl)-4-(2-difluoromethoxyphenyl)-1,4-dihydropyridine (60–62 Ci/mmol) as a radioactive ligand.

Assay of DHP receptor binding in membrane preparations. Sarcoplasmic reticulum membranes (100–150 μg of protein) were incubated for 20 min at 20°C with indicated concentrations of radioactive ligand in a final volume of 1 ml containing 20 mM Tris · HCl (pH 7.4), 1 mM $CaCl_2$, and 10 μM D-*cis*-diltiazem. The reaction was terminated by pipetting 0.4-ml duplicate aliquots of the radioactive mixture into 3 ml of ice-cold 20 mM Tris · HCl, pH 7.4. The samples were filtered rapidly under vacuum through GF/C glass fiber filters. Each filter was then washed twice with 3 ml of the same buffer. The radioactivity trapped on filters was measured by liquid scintillation spectrometer. The specific binding of a radioactive ligand was defined as total binding minus the binding obtained in the presence of a 500-fold molar excess of the related nonradioactive DHP derivative.

Competitive radioligand assay. DHP derivatives obtained have been evaluated for their biological activity in competitive binding studies with **3a** as a specific radioactive ligand essentially as it was specified above. In brief, membranes were incubated with 1 nM **3a** for 1.5 h at 20°C in the presence of up to 50 nM of synthesized nonradioactive DHP derivatives. Competitive displacement data were used for the determination of inhibitory dose 50 (ID_{50}), the concentration of the compound that produced 50% inhibition of the binding of **3a** to membranes as compared to the untreated control.

Preparation of anti-DHP antibodies. New Zealand White rabbits (2.5–3 kg body wt) were used for immunization with DHP–BSA conjugate **9.** Antigen was emulsified with Freund's complete adjuvant at 1 mg/ml. Animals were injected into the hind footpads with 0.2 ml of the emulsion. One month later the injection was repeated subcutaneously in four or five sites with a similar amount of antigen made up in Freund's incomplete adjuvant. The boost immunization was made subcutaneously in two sites with a suspension of antigen in saline (1.25 mg/ml, 0.3 ml per animal). Blood was collected 8 days after the last immunization by cardiac puncture. Antiserum was stored in 2-ml aliquots at −35°C.

Radioimmunoassay. Dose-dependent binding of whole rabbit antiserum was measured in phosphate-buffered saline. Antiserum at the indicated dilutions was incubated for 18 h at 4°C with 1.8 nM compound **3a** as a radioactive antigen in a final volume of 1.2 ml. Bovine serum albumin (5 mg) was added to each assay tube (with the exception of 1 : 25 and 1 : 50 dilutions of antiserum). Ten microliters of goat anti-rabbit globulin (1.8 μg protein) was added to each assay mixture. After an additional 4 h at 4°C, the antibody–antigen complexes were separated from unbound **3a** by polyethylene glycol precipitation. Each of two 0.5-ml aliquots of

assay mixtures was pipetted into 3.5 ml of ice-cold 11.4% polyethylene glycol solution containing 11.4 mM $MgCl_2$ and 20 mM Tris · HCl, pH 7.4. After incubation for 15 min at 4°C, samples were filtered through GF/C filters. Each filter was rapidly washed twice with 3.5 ml of ice-cold precipitation buffer and radioactivity remaining on the filters was counted.

Immunotitration of the radioactive antigen was carried out in the same fashion at a fixed antiserum dilution of 1 : 250 and increasing concentrations of compound **3a** (1 to 6 nM). Nonspecific binding of radioactive antigen was determined in the presence of a 500-fold molar excess of **3b**.

Isolation of DHP-directed antibodies. Immune serum (25 ml) was diluted twofold with phosphate-buffered saline and incubated batchwise for 2 h at 20°C with 5 ml of DHP-Sepharose 4B prepared by method a. The resin was then packed in a column and washed with 20 mM Tris · HCl (pH 7.4) containing 1 M NaCl until the optical density of the flow-through fractions recorded at 280 nm approached zero. Bound anti-DHP antibodies were eluted with 0.1 M glycine · HCl buffer (pH 2.6) containing 1 M NaCl. The pooled peak fraction was rapidly neutralized with 1 M Tris, then dialyzed against Tris-buffered saline, and finally concentrated by ultrafiltration on Amicon PM10 membranes under argon.

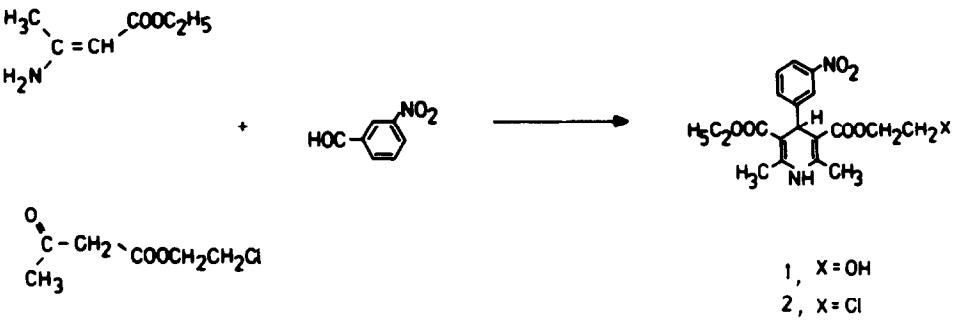
Protein concentrations were determined by the Hartree method (*11*) using BSA as standard.

RESULTS

Bossert and Vater initially reported (*12*) synthesis of nifedipine, the parent drug of the DHP family. A number of nifedipine congeners including various aryl- and alkoxycarbonyl-substituted ones were synthesized and tested for the hypotensive potency. These studies demonstrated that both these sites of the DHP molecule are important for biological activity, the most potent being dihydropyridines with electron-withdrawing substituents (NO_2, CF_3, etc.) in the *meta*- or *ortho*-positions of the aromatic ring as well as those containing structurally nonsymmetric alkoxycarbonyl groups at the positions 3 and 5 of the dihydropyridine ring (*13–15*). Therefore, the structure of the key compound **1** used in this work was designed so that the attachment of various functional groups proceeded at the single alkoxycarbonyl group of DHP molecule.

Compound **1** was isolated as a major by-product formed most probably by the hydrolysis of the initial primary target, compound **2.** The latter was formed by Hantzsch condensation of ethyl 3-aminocrotonate, *m*-nitrobenzaldehyde and 2-chloroethyl acetoacetate according to Scheme 1. Compounds **1** and **2** were isolated from the reaction mixture in about 1 : 10 molar ratio. Alternatively, **1** was purified from minor impurities with close R_f values by reversible silylation with *N*-trimethylsilyldiethylamine followed by chromatographic separation of the relative trimethylsilyl derivative of **1** and regeneration of the target key compound **1** by mild acidification.

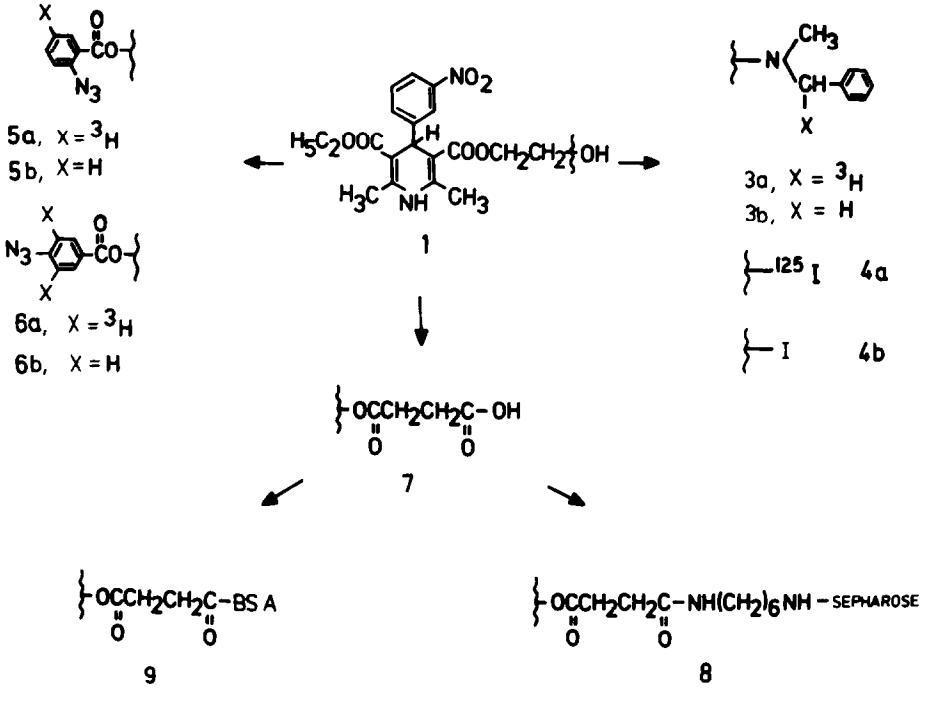
Acylation of **1** with acetic anhydride gave the corresponding acetate. Similarly, treatment of **1** with methane sulfonyl chloride afforded the respective mesylate in



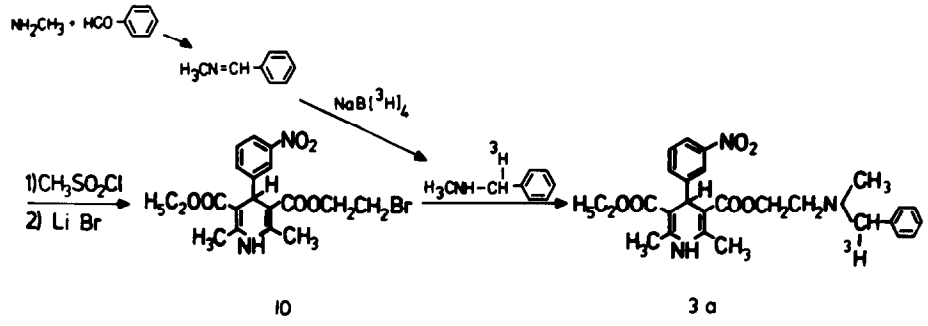
SCHEME 1

a good yield. These substitutions on a hydroxy moiety of **1** demonstrate the relatively high reactivity of this site of the molecule which is hence a suitable starting material for the introduction of various functional groups.

The synthetic strategy for essentially all the DHP derivatives under consideration was based on the reactions of the hydroxy group of the key compound **1** as outlined in Scheme 2. The series of synthesized DHP derivatives includes (i) compounds **3a** and **4a** suitable for radioligand binding assay of the DHP receptor as well as for evaluation of the biological activity of other DHP derivatives by



SCHEME 2



SCHEME 3

competitive binding studies; (ii) two tritium-labeled photoactivable derivatives **5a** and **6a** which are potential photoaffinity probes for the covalent labeling of the DHP receptor; (iii) DHP hemisuccinate **7** suitable for the covalent attachment to aminohexyl-Sepharose thus resulting in affinity resin **8** which may be applied for the purification of the DHP receptor from detergent-solubilized membrane preparations; and (iv) covalent DHP–BSA conjugate **9** as an antigen for the production of antibodies specific for the DHP calcium entry blockers.

Compounds **3–6** shown in Scheme 2 were initially synthesized as nonradioactive congeners in preparative scale. This was done in an effort to provide both optimal conditions of the reactions and definitive evidence of a structural assignment of the compounds obtained. Corresponding nonlabeled derivatives were used as chromatographic standards for comparison with radioactively labeled products as well.

Compound **3** is the ethoxycarbonyl derivative of nicardipine (YC-93) (*16*), one of the most potent DHP drugs. It was obtained in good yield in tritium-labeled form **(3a)** by the substitution of haloid atom in compound **10** with labeled benzylmethylamine, performed in line with Scheme 3. The reaction proceeds under relatively mild conditions when bromide is to be substituted and, therefore, bromo derivative **10** was obtained from **1** by treatment of the relative mesylate with LiBr in absolute acetone. Molar radioactivity of the target compound **3a** (8.1 Ci/mmol) depended on the specific radioactivity of the sodium boro[^{3}H]hydride preparation used (8 Ci/mmol) since only one unexchangable tritium atom was attached to *N*-benzylidenemethylamine.

Figure 1 shows the Scatchard plot of the binding of labeled compound **3a** to rabbit skeletal muscle membranes. It is seen that the probe interacts with the single class of binding sites, the dissociation constant being 4.7 nM in the presence of 10 μM D-*cis*-diltiazem added to enhance the affinity of the receptor to the ligand (*10, 17*). Compound **3a** was used further in competitive binding assay for evaluation of the potency of other target compounds obtained (Table 1).

Substitution of the hydroxy group of the key compound **1** for a haloid atom may be used for the preparation of highly radioactive [^{125}I]iodo DHP derivative **4a.** Nonradioactive analog of such a compound **(4b)** was obtained from the mesylate of **1** in a good yield. Competitive binding assay (Table 1) demonstrated that this

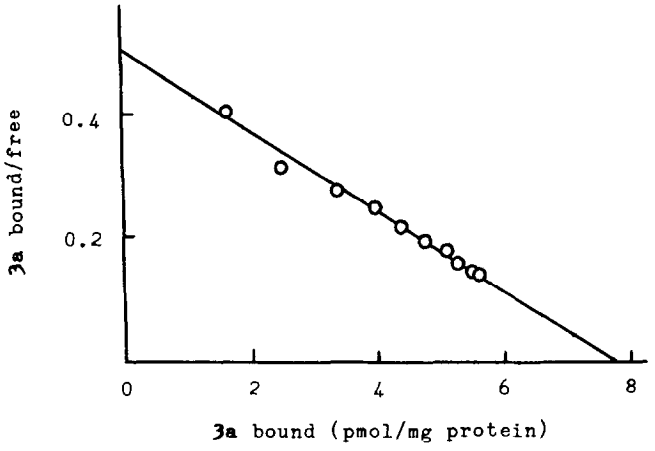


FIG. 1. Scatchard analysis of equilibrium binding of radioactive probe **3a** to DHP receptor in rabbit skeletal muscle sarcoplasmic reticulum. Dissociation constant (K_d) of the **3a**-receptor complex is 4.7 nM; maximal binding capacity is 7.6 pmol/mg protein.

derivative possesses an affinity high enough to detect the DHP receptor in minute amounts when it is in radioactive form. Indeed, ^{125}I-labeled derivative **4a** was shown to be able to detect subfemtomole quantities of the receptor in skeletal muscle homogenate.

DHP calcium entry blockers interact with DHP receptors in various plasma membranes with high specificity. Extension of this series of calcium channel probes to the photoactivable derivatives may yield additional information about the molecular organization of the DHP binding site. A number of such photoaffinity probes with the light-sensitive arylazido group attached to the active site of DHP molecule at the alkoxycarbonyl moiety were prepared by the esterification of the hydroxy group of the compound **1** with azidobenzoic acid through mixed

TABLE 1

Comparative Potency of Novel DHP Derivatives

Compound	ID_{50} (M)	K_d[a] (M)
3a	—	4.7×10^{-9}
4a	—	1.5×10^{-9}
4b	1.7×10^{-9}	—
5a	—	3.8×10^{-9}
5b	3.9×10^{-9}	—
6a	—	3.5×10^{-9}
6b	4.0×10^{-9}	—
7	7.2×10^{-9}	—

[a] In all cases Scatchard plots were linear, which is indicative of a single class of noninteracting binding sites.

anhydride formed in the presence of ethyl chloroformate. The accessibility of the receptor moiety to nitrene generated by uv light irradiation may depend strongly on the position of the azido group in aromatic ring. Therefore, two derivatives were prepared with azido group attached to the *ortho-* or *para*-position (compounds **5** and **6,** respectively). The corresponding radioactively labeled arylazido adducts **5a** and **6a** were synthesized from the related labeled azidobenzoic acids prepared by catalytic hydrogenation with tritium gas of 3-iodo-2-aminobenzoic or 3,5-diiodo-4-aminobenzoic acids, respectively.

In competitive binding studies with compound **3a** both photosensitive nonlabeled derivatives **5b** and **6b** demonstrated a high level of biological activity (see Table 1). ID_{50} values obtained correlated well with K_d values determined from Scatchard plots of the binding of respective radioactive compounds to rabbit skeletal muscle membranes in dark. The application of these photoactivable probes for the photoaffinity labeling of the DHP receptor will be described elsewhere.

The reversibility of the receptor interaction with DHP calcium entry blockers as well as the high affinity of the receptor to DHPs suggest that the receptor protein may be purified by affinity chromatography. Therefore, the synthesis of affinity resin has been undertaken. The choice of the ligand was based on the general observation that an amide bond between the hydroxy ligand and the spacer arm is more resistant to hydrolysis than an ester linkage. Accordingly, DHP hemisuccinate **7** was selected as a convenient biospecific ligand which could be connected to the amino spacer arm by amide bond (Scheme 2). DHP hemisuccinate **7** exhibits specific and reversible binding to the receptor with an affinity constant optimal for biospecific chromatography (Table 1). AH-Sepharose 4B was selected as a matrix since, firstly, the exclusion limit of this resin is high enough to make the interior of the resin beads available for the attachment of **7,** thus providing high binding capacity of affinity resin for solubilized receptor. Secondly, a six-carbon long spacer arm of AH-Sepharose allows the problem of steric interference between the matrix and receptor molecules to be overcome. On the other hand, the increased length of alkoxycarbonyl side chain in the DHP molecule does not influence significantly the biological activity of the drug (cf. nisoldipine (*18*)).

Coupling of DHP hemisuccinate **7** on AH-Sepharose was performed with two different, currently used condensation agents. In method a, water soluble carbodiimide was applied with success to give an affinity resin containing ca. 3.5 μmol DHP/ml of packed gel as shown by the difference in amounts of hemisuccinate **7** taken into the reaction and that released from the reaction mixture. Even more favorable was method b, which was based on the usage of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline as a condensation agent (*19*). This method afforded the affinity resin at 2–10 μmol/ml gel depending on the concentration of the ligand used for the reaction. In all cases, the excess of reactive amino groups of the matrix was blocked with acetic acid.

In a number of experiments using Chapso-solubilized transverse tubule membranes of rabbit skeletal muscle it was shown that the degree of substitution of AH-Sepharose at about 3 μmol/ml gel is optimal for the preparation of a convenient affinity resin. Direct binding studies revealed that such a resin interacts spe-

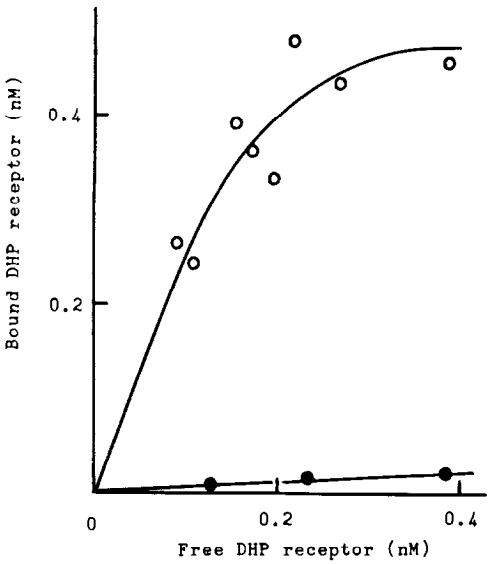


FIG. 2. Binding of Chapso-solubilized DHP receptor to DHP-Sepharose 4B **(8)**. Portions (0.05 ml) of affinity resin were incubated batchwise for 3.5 h with increasing amounts of Chapso extract in constant volume of 2.5 ml. After the resin was precipitated by centrifugation, the amount of DHP receptor was determined in supernatant (*free DHP receptor*). The concentration of *bound DHP receptor* was calculated as the difference between the amount of the receptor taken into the reaction and *free receptor* values (○). (●) Binding curve for affinity resin with DHP groups preoxidized to pyridine groups by brief treatment with 0.5% H_2O_2.

cifically and reversibly with the solubilized DHP receptor with a K_d value of 1.2 nM, the maximum binding capacity being ca. 120 pmol/ml gel (Fig. 2). It follows, therefore, that only a minor portion of the ligand molecules attached to Sepharose beads is accessible to the solubilized receptor. This is supposed to be due to the size of the receptor-detergent micelles. Nevertheless, the preparative levels of the receptor immobilization by the affinity resin were achieved for 10% (v/v) suspension of the DHP-Sepharose in detergent extract of the membranes (data not shown). Detailed investigation of factors affecting the binding and elution of the receptor from various detergent extracts will be presented elsewhere.

DHP hemisuccinate **7** reactions were extended to the preparation of DHP–protein conjugate which is a potential antigen for the production of anti-DHP antibodies. Bovine serum albumin was used as a carrier protein for the preparation of DHP–BSA conjugate **9** with water soluble carbodiimide as a condensation agent. The resulting conjugate contained ca. 0.2 μmol DHP/mg protein and was virtually insoluble in water.

DHP–BSA was subsequently used for antisera production in rabbits. The evaluation of the affinity and specificity of antiserum obtained was carried out by radioimmunoassay with **3a** as a radioactive antigen. The antibody–antigen complexes formed after incubation with **3a** were separated as secondary complexes

with goat anti-rabbit globulin by polyethylene glycol coprecipitation with BSA as a carrier protein.

Radioimmune titer test has demonstrated that 50% of **3a** was bound from 1.8 nM solution at 1 : 300 serum dilution. Preimmune serum revealed no specific binding to **3a** under the same conditions. The immunotitration of radioactive antigen performed at postimmune antiserum dilution of 1 : 250 (Fig. 3) resulted in an average apparent K_d value for DHP–antibody complex of 1.7 nM.

To separate the fraction of DHP-specific serum globulins, the affinity chromatography of antiserum on DHP-Sepharose **8** was used. Anti-DHP antibodies eluted with glycine · HCl buffer at pH 2.6 retained the affinity and specificity to antigen as demonstrated by radioimmunoassay.

DISCUSSION

A series of novel DHP congeners obtained in this study encompasses a broad spectrum of substituents indispensable for identification, characterization, and isolation of the DHP receptor, a putative calcium channel candidate. Synthetic methods presented in this paper are superior to those published elsewhere in obvious simplicity. The choice of hydroxy derivative **1** as the key starting compound provided the introduction of radioactive groups (i.e., [^{3}H]benzylmethylamino or [^{125}I]iodo) and photoactivable substituents (i.e., [^{3}H]arylazido) as well as spacer arm close to the active site of the molecule. All the derivatives obtained possess a biological potency high enough to be applied as specific molecular tools of the receptor investigation.

Nicardipine (YC-93) has been initially synthesized (*16*) as a water soluble dihydropyridine vasodilator with high biological potency and bioavailability. These properties were expected to improve general binding characteristics of DHP

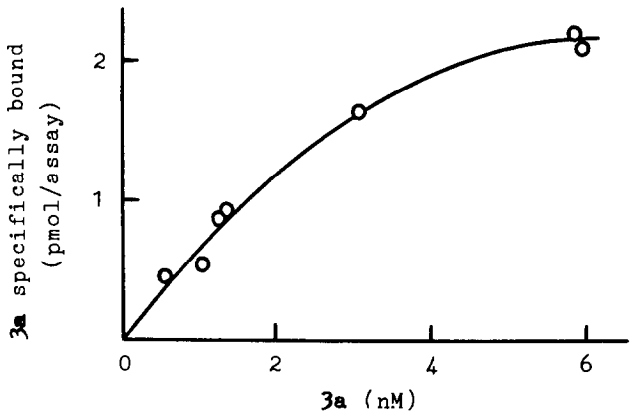


FIG. 3. Immunotitration of radioactive antigen **3a** at constant immune antiserum dilution of 1 : 250. Data were corrected for nonspecific binding of **3a** which accounted for ca. 20% of the total binding.

probes currently used in the radioligand assay of the DHP receptor, first of all due to the decrease of a hydrophobicity of the molecule. Therefore, a synthesis of radioactive ethoxycarbonyl congener **3a** of nicardipine was carried out. Molar radioactivity of **3a** depended on the radioactivity of sodium boro[^{3}H]hydride used for the reduction of the intermediate aldimine (Scheme 3). In the case described under Experimental Procedures it was about 8 Ci/mmol, which was sufficient to detect membrane-bound DHP receptor in amounts as small as 50–100 fmol per assay. Compound **3a** was used further in competitive binding experiments as a radioactive ligand for the estimation of potencies of other DHP derivatives obtained (Table 1).

In the related investigations, the compound 2,6-dimethyl-3-ethoxycarbonyl-5-[(2-aminoethoxy)carbonyl]-4-(2-trifluoromethylphenyl)-1,4-dihydropyridine was used as starting material for synthesis of 1-(3-[^{125}I]iodo-4-hydroxyphenyl)-3-oxopropyl adduct ([^{125}I]iodipine) (*20*) and ^{3}H-labeled 4-azidobenzoate photoactivable derivative ([^{3}H]azidopine) (*21*), potent high affinity ligands for the study of DHP receptors. Essentially simpler methods underlie the preparation of respective derivatives described in this work, including the synthesis of starting key hydroxy compound **1.**

The substitution of the reactive hydroxy group of **1** with [^{125}I]iodo one was anticipated to result in the formation of related [^{125}I]iodo derivative **4a** which would be essentially more resistant to hydrolysis in biological media than iodipine. The compound **4a** is about threefold less potent than iodipine as follows from comparison of their K_d values. However, in contrast to iodipine, **4a** is stable at least for 2 days when incubated with skeletal muscle microsomes. So, one can conclude that compound **4a** is a convenient alternative probe for the DHP receptor, especially in cases when its high molar radioactivity (ca. 2000 Ci/mmol) is important, i.e., for rapid autoradiography and/or receptor identification at low densities of DHP-binding sites.

As a part of a study, photoactivable derivatives **5** and **6** were prepared by the esterification of compound **1** with a mixed anhydride formed on the carboxyl group of an azidobenzoic acid treated with ethyl chloroformate. As followed from comparison of K_d values, the resulted photoaffinity probes **5** and **6** were about 10 times less potent than azidopine. This is supposed to be due to the nature of 4-aryl substituent of these molecules which affect significantly the biological activity of DHP calcium antagonists (*13, 14*). This difference, however, is not critical and, in fact, compounds **5a** and **6a** fulfill the requirements applied to photoaffinity probes. The derivatives obtained interact reversibly and specifically with a certain class of binding sites related to DHP receptor. Relatively low K_d values as well as high molar radioactivity of these probes and variable location of photosensitive azido groups with respect to the aromatic ring allow application of **5a** and **6a** for the photoaffinity labeling studies of the DHP receptor. The results of such an investigation are to be published elsewhere.

Introduction of a free carboxyl group on to the side chain of key compound **1** to form the related hemisuccinate **7** does not cause significant reduction in biological activity. This hemisuccinyl-substituted derivative of DHP could be a useful intermediate for coupling of DHP molecules to various insoluble matrices as well as to

carrier proteins via amide bonds formation with free amino groups catalyzed by water soluble carbodiimide or *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline as a condensation agent. This approach is more favorable with respect to the one described by Biswas and Rogers (*22*), who used carboxynifedipine as an intermediate for coupling to hexylamine agarose through acid chloride formed after the treatment of the ligand with oxalyl chloride. The DHP-Sepharose **8** prepared as described under Experimental Procedures is characterized by high affinity to the solubilized receptor (Fig. 2) that underlies its successful application for the isolation of the DHP-binding protein (*23*).

The DHP hemisuccinate **7** was used also for preparation of DHP–BSA conjugate **9** suitable for the production of anti-DHP antibodies in rabbits. This approach differs from that of Campbell *et al.* (*24*), who used the 4-(2-isothiocyanatophenyl) analog of nifedipine for coupling to BSA via the reactive group in the 4-aryl substituent of the molecule. In contrast, DHP–BSA conjugate **9** in this work was prepared without this biologically active site of the molecule being involved in the coupling reaction. The anti-DHP antibodies obtained were characterized by very high affinity to DHP congeners (Fig. 3) and could be selectively isolated by DHP-Sepharose affinity chromatography.

In conclusion, hydroxy compound **1** described herein is certainly a convenient starting material for the preparation of DHP derivatives with various specific groups attached to or near the active site of the molecule without loss of biological activity. The radioactive and photoactivable DHP derivatives and those immobilized on Sepharose beads, as well as anti-DHP antibodies, comprise a series of valuable pharmacological tools for the investigation of the DHP receptors related to long-lasting calcium channels of biological membranes.

ACKNOWLEDGMENTS

I express my gratitude to Dr. S. M. Dudkin for critically reviewing this manuscript. I also thank M. V. Mikhailushkina for excellent editorial assistance and A. V. Petrov for illustrations.

REFERENCES

1. JANIS, R. A., AND TRIGGLE, D. J. (1983) *J. Med. Chem.* **62,** 775–785.
2. EHLERT, F. J., ITOGA, E., ROESKE, W. R., AND YAMAMURA, H. I. (1982) *Biochem. Biophys. Res. Commun.* **104,** 937–943.
3. FERRY, D. R., AND GLOSSMANN, H. (1982) *FEBS Lett.* **148,** 331–337.
4. JANIS, R. A., MAURER, S. C., SARMIENTO, J. G., BOLGER, G. T., AND TRIGGLE, D. J. (1982) *Eur. J. Pharmacol.* **82,** 191–194.
5. HESS, P., LANSMAN, J. B., AND TSIEN, R. W. (1984) *Nature (London)* **311,** 538–544.
6. NILIUS, B., HESS, P., LANSMAN, J. B., AND TSIEN, R. W. (1985) *Nature (London)* **316,** 443–446.
7. NOWYCKY, M. C., FOX, A. P., AND TSIEN, R. W. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2178–2182.
8. GOA, K. L., AND SORKIN, S. M. (1987) *Drugs* **33,** 123–155.
9. ROSEMBLATT, M., HIDALGO, C., VERGARA, C., AND IKEMOTO, M. (1981) *J. Biol. Chem.* **256,** 8140–8148.

10. GLOSSMANN, H., AND FERRY, D. R. (1983) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **323,** 279–291.
11. HARTREE, E. F. (1972) *Anal. Biochem.* **48,** 422–427.
12. BOSSERT, F., AND VATER, W. (1971) *Naturwissenschaften* **58,** 578.
13. LOEV, B., GOODMAN, M. M., SNADER, K. M., TEDESCHI, R., AND MACKO, E. (1974) *J. Med. Chem.* **17,** 956–965.
14. BOSSERT, F., HORSTMANN, H., MEYER, H., AND VATER, W. (1979) *Arzneim.-Forsch./Drug Res.* **29,** 226–229.
15. MANNHOLD, R., RODENKIRCHEN, R., AND BAYER, R. (1982) *Progr. Pharmacol.* **5,** 25–52.
16. IWANAMI, M., SHIBANUMA, T., FUJIMOTO, M., KAWAI, R., TAMAZAWA, K., TAKENAKA, T., TAKAHASHI, K., AND MURAKAMI, M. (1979) *Chem. Pharm. Bull.* **27,** 1426–1440.
17. DEPOVER, A., MATLIEB, M. A., LEE, S. W., DUBE, G. P., GRUPP, I. L., GRUPP, G., AND SCHWARTZ, A. (1982) *Biochem. Biophys. Res. Commun.* **108,** 110–117.
18. KASS, R. S. (1982) *J. Pharm. Exp. Ther.* **223,** 446–456.
19. BOSCHETTI, E., CORGIER, M., AND GARELLE, R. (1978) *Biochimie* **60,** 425–427.
20. FERRY, D. R., AND GLOSSMANN, H. (1984) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **325,** 186–189.
21. FERRY, D. R., ROMBUSCH, M., GOLL, A., AND GLOSSMANN, H. (1984) *FEBS Lett.* **169,** 112–118.
22. BISWAS, C. J., AND ROGERS, T. B. (1986) *Biochem. Biophys. Res. Commun.* **134,** 922–927.
23. SOLDATOV, N. M. (1988) *Eur. J. Biochem.* **173,** 327–338.
24. CAMPBELL, K. P., SHARP, A., STROM, M., AND KAHL, S. D. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2792–2796.