Synthesis and Pharmacological Properties of Azido Derivatives of 1,5-Benzothiazepine Ca Antagonist

Hiroshi NARITA,*[,a] Mitsunori GAINO,[b] Tatsuo SUZUKI,[a] Hideyasu KUROSAWA,[a] Hirozumi INOUE[b] and Taku NAGAO[a]

Biological Research Laboratory,[a] Organic Chemistry Research Laboratory,[b] Tanabe Seiyaku Co., Ltd., Kawagishi 2-2-50, Toda-shi, Saitama 335, Japan. *Received July 27, 1989*

Since azido derivatives of 1,5-benzothiazepine Ca antagonist available for photoaffinity labeling are required for further studies of voltage-sensitive Ca channels, we synthesized 3-(*p*-azidobenzoyloxydeacetyl)- and 3-(4-azidobutyryloxydeacetyl)-diltiazem, and studied their pharmacological properties. Both azido compounds showed similar relaxing actions to diltiazem in K^+-depolarized dog arteries. They also showed a similar increasing action to diltiazem, but less potent, on the coronary and vertebral blood flow in the anesthetized dog. Moreover, their negative inotropic effects in the guinea pig papillary muscle were similar to or slightly more potent than that of diltiazem under physiological conditions, but were less potent when studied in K^+ depolarizing solution. A radioligand binding study in rat skeletal muscle microsomes revealed that the azido derivatives had similar properties to diltiazem, but the nonspecific binding of 3-(*p*-azidobenzoyloxydeacetyl)-diltiazem was too high to allow estimation of its K_D and B_{max} values. In conclusion, we synthesized azido derivatives of diltiazem which were considered to share a common binding site on the voltage-dependent Ca channel with diltiazem in skeletal muscle microsomes and in vascular smooth muscle.

Keywords diltiazem; 1,5-benzothiazepine; calcium antagonist; azido derivative; photoaffinity labeling; radioligand binding; 3-(*p*-azidobenzoyloxydeacetyl)-diltiazem; 3-(4-azidobutyryloxydeacetyl)-diltiazem

Recent developments in molecular biology have led to elucidation of the primary structures of various kinds of receptors and ion channels as well as their functions and three dimensional structures. Concerning the voltage-dependent L type Ca channel, Tanabe *et al.* elucidated its primary structure using the T system of skeletal muscle.[1]

1,4-Dihydropyridine derivatives and phenylalkylamine derivatives as well as 1,5-benzothiazepine derivatives are representative compounds acting on the voltage-dependent L type Ca channel. The results of radioligand binding studies indicated that these three chemical groups had individual binding sites on the voltage-dependent Ca channel,[2,3] but at the same time each group allosterically influences the binding of the others.[4,5] Furthermore, 1,4-dihydropyridines and phenylalkylamines were reported to bind to different sites on the same α_1 subunit of the Ca channel protein by the technique of photoaffinity labeling[5-7] using their azido derivatives, azidopine[8] and LU49888.[9] 1,5-Benzothiazepine derivatives are also speculated to have their own binding site on the same α_1 subunit, but the absence of compounds available for photoaffinity labeling prevents further investigation.[10]

Therefore we have synthesized two azido derivatives of 1,5-benzothiazepine Ca antagonist expected to be useful for photoaffinity labeling and studied their pharmacological and radioligand binding properties. Since these two compounds, 3-(*p*-azidobenzoyloxydeacetyl)-diltiazem (azidobenzoyl-diltiazem) and 3-(4-azidobutyryloxydeacetyl)-diltiazem (azidobutyryl-diltiazem), exhibited similar pharmacological and radioligand binding properties to diltiazem, they were considered to share a common binding site on the voltage-dependent Ca channel with diltiazem. However, nonspecific binding of azidobenzoyl-diltiazem was too high to allow estimation of its K_D and B_{max} values. Thus azidobutyryl-diltiazem seemed to be better probe for photoaffinity labeling.

Experimental

The chemical structures of azidobenzoyl-diltiazem and azidobutyryl-diltiazem as well as diltiazem are shown in Fig. 1. Both compounds have acyloxy substituents carrying azido group at position 3 of the diltiazem structure, and are 2*S*,3*S* isomers.

Drugs Diltiazem (Tanabe Seiyaku Co., Ltd.) was used as a reference compound. Diltiazem and azidobutyryl-diltiazem were dissolved in deionized water for the *in vitro* experiment and in 0.9% NaCl solution for the *in vivo* experiment. Azidobenzoyl-diltiazem was dissolved in deionized water and diluted with 0.9% NaCl solution or deionized water. The volume of administration was adjusted to 0.01 or 0.03 ml/kg and to 0.1 ml in the *in vivo* and *in vitro* experiments, respectively.

For the radioligand binding study, [^{3}H]nitrendipine (spec. act. 2.73 TBq/mmol) was purchased from New England Nuclear, and [methyl-^{3}H]diltiazem (spec. act. 5.99 TBq/mmol) was purchased from Amersham. [*N*-Methyl-^{3}H]azidobutyryl-diltiazem (spec. act. 2.66 TBq/mmol) and [*N*-methyl-^{3}H]azidobenzoyldiltiazem (spec. act. 3.07 TBq/mmol) were prepared by Amersham from our synthesized azido compounds. They were dissolved in 10% ethanol solution at the concentration of 10^{-4} M and then diluted with deionized water.

Other chemicals were purchased from conventional commercial sources.

Synthesis of Azido Derivatives 2*S*,3*S*-3-(4-Azidobenzoyl)oxy-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one (**2**): Dicyclohexylcarbodiimide (1.03 g) was added to a mixture of 2*S*,3*S*-5-[2-(dimethylamino)ethyl]-2,3-dihydro-3-hydroxy-2-

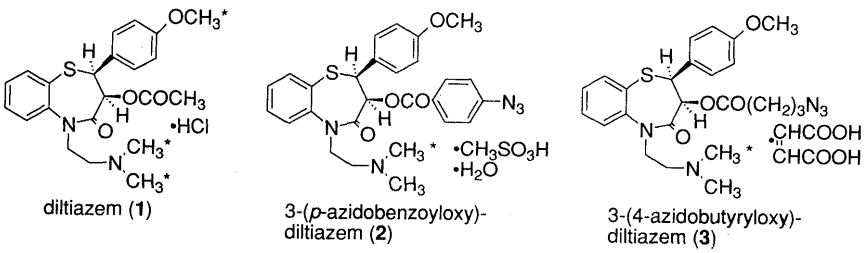

Fig. 1. Structures

* Indicates the position of radiolabeling.

(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one (**4**) (1.5 g), 4-azidobenzoic acid (980 mg), and 1-hydroxybenzotriazole hydrate (150 mg) in dimethylformamide (DMF) (40 ml) under ice-cooling. The reaction mixture was stirred at room temperature for 48 h, poured into ice-water, and extracted with AcOEt. The extracts were combined, washed with water, 5% $NaHCO_3$, and saturated aqueous NaCl successively, dried over Na_2SO_4, and concentrated. The residual oil was purified by column chromatography (silica gel, eluted with $CHCl_3$–MeOH–EtOH (98 : 1 : 1)) and the obtained oil (1.25 g) was converted into the methanesulfonate and recrystallized from iso-PrOH–iso-Pr_2O to give **2**·methanesulfonate·hydrate, colorless needles, mp 115—117.5 °C (770 mg, yield: 30.3%). *Anal.* Calcd for $C_{27}H_{27}N_5O_4S \cdot CH_4O_3S \cdot H_2O$: C, 53.24; H, 5.27; N, 11.09; S, 10.15. Found: C, 53.17; H, 5.27; N, 11.07; S, 10.09. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3700, 3430, 2410, 2260, 2130, 1720, 1675, 1600. ^{1}H-NMR ($CDCl_3$) δ: 1.97 (2H, s, H_2O), 2.75 (3H, s, CH_3SO_3H), 2.94 (6H, br s, NCH_3), 3.1—3.9 (2H, m, CH_2N), 3.84 (3H, s, OCH_3), 4.1—4.7 (2H, m, CH_2N), 5.12 (1H, d, J=7.9 Hz), 5.32 (1H, d, J=7.9 Hz), 6.7—7.8 (12H, m, aromatic H). $[\alpha]_D^{20}$ −20.0° (c=0.185, MeOH).

2*S*,3*S*-3-(4-Azidobutyryl)oxy-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one (**3**): A solution of 4-bromobutyryl chloride (1.06 g) in CH_2Cl_2 (5 ml) was added to a solution of **4**·hydrochloride (2.05 g) in DMF (25 ml) and the mixture was stirred at 50 °C for 24 h, then concentrated under reduced pressure. The residual oil was dissolved in $CHCl_3$, washed with saturated aqueous NaCl, dried over Na_2SO_4, and concentrated to give the 3-(4-bromobutyryl)oxy compound (**5**)·hydrochloride (1.7 g, yield: 60.8%), as a white powder. ^{1}H-NMR ($CDCl_3$) δ: 1.84 (2H, quintet, J=7 Hz, CH_2), 2.34 (2H, t, J=7Hz, CH_2CO), 2.86 (6H, s, NCH_3), 3.39 (2H, t, J=7Hz, CH_2Br), 3.0—3.7 (2H, m, CH_2N), 3.82 (3H, s, OCH_3), 4.1—4.8 (2H, m, CH_2N), 5.00 (1H, d, J=7.9 Hz), 5.13 (1H, d, J=7.9 Hz), 6.65—7.9 (8H, m, aromatic H). IR ν_{max}^{Nujol} cm^{-1}: 2700—2000, 1740, 1680, 1640.

5·Hydrochloride (1.27 g) was reacted with NaN_3 (450 mg) in dimethyl sulfoxide (DMSO) (10 ml) at 50 °C for 24 h. The reaction mixture was poured into a mixture of cracked ice, 5% $NaHCO_3$, and Et_2O, and extracted with Et_2O. The extracts were combined, washed with water, dried over Na_2SO_4, and concentrated. The obtained oil was converted to the maleate and recrystallized from AcOEt to give **3** maleate, colorless needles, mp 125.5—127 °C (dec.) (523 mg, yield: 38.3%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2460, 2330, 2105, 1735, 1700, 1680, 1620, 1605. ^{1}H-NMR ($CDCl_3$) δ: 1.66 (2H, quintet, J=6.7 Hz, CH_2), 2.26 (2H, t, J=6.7 Hz, CH_2CO), 2.88 (6H, s, NCH_3), 3.16 (2H, t, J=6.7 Hz, CH_2N_3), 2.7—3.7 (2H, m, NCH_2), 3.82 (3H, s, OCH_3), 4.0—4.7 (2H, m, CH_2N), 5.00 (1H, d, J=7.9 Hz), 5.13 (1H, d, J=7.9 Hz), 6.25 (2H, s, maleic acid), 6.7—7.85 (8H, m, aromatic H). *Anal.* Calcd for $C_{24}H_{29}N_5O_4S \cdot C_4H_4O_4$: C, 56.08; H, 5.55; N, 11.68; S, 5.35. Found: C, 56.33; H, 5.53; N, 11.40; S, 5.44. $[\alpha]_D^{20}$ +70.0° (c=0.91, MeOH).

Increasing Action on Arterial Blood Flow Nine male mongrel dogs weighing 11.0—27.0 kg were anesthetized with sodium pentobarbital (30 mg/kg, i.v. and 3—4 mg/kg/h, i.v. infusion) and artificially respirated (15 ml/kg/stroke, 20 strokes/min, model 100, Takashima, Tokyo). Arterial blood flow was measured by attaching a flow probe of an electromagnetic flowmeter (MF-27, Nihon Koden, Tokyo) to the left circumflex coronary artery after thoracotomy at the fourth intercostal space, or the right vertebral artery. In addition, a polyethylene catheter was inserted into the arteries for drug administration at the peripheral side of the flow probe.

All measurements, including systemic blood pressure and heart rate, were simultaneously recorded on a Linearcorder (WR-3701, Graphtec, Tokyo).

Azido derivatives as well as diltiazem increased the coronary and the vertebral blood flow (Fig. 2). Diltiazem increased the coronary blood flow at doses of 0.3 μg/kg, i.a. or more, and increased the vertebral blood flow at doses of 0.1 μg/kg, i.a. or more. The azido derivatives showed similar but slightly less potent dose-related increasing actions as compared with diltiazem on both blood flows. Their dose–response curves were parallel to that of diltiazem in the case of the vertebral blood flow, but were slightly shallower than that of diltiazem in the case of the coronary blood flow.

Relaxing Action on the Isolated Artery After sodium pentobarbital anesthesia (30 mg/kg, i.v.) 6 mongrel dogs weighing 10.0—16.4 kg were killed by intravenous administration of saturated KCl solution. The brain and the heart were excised and stocked overnight in physiological salt solution (PSS, NaCl; 147.2 mM, KCl; 5.4 mM, $MgCl_2$; 1.0 mM, $CaCl_2$; 2.2 mM, $NaHCO_3$; 14.9 mM, dextrose; 5.6 mM) at 4 °C. The trunk of the left coronary artery, and the basilar artery were isolated and cleaned in ice-cold PSS, and then ring segments of 5 mm long were prepared. A ring segment was suspended in an organ bath filled with oxygenated PSS (95% O_2 and 5% CO_2, 37 °C). Isometric tension was measured with a strain gauge transducer (UL-10, Minebea, Tokyo) and recorded on a recorder

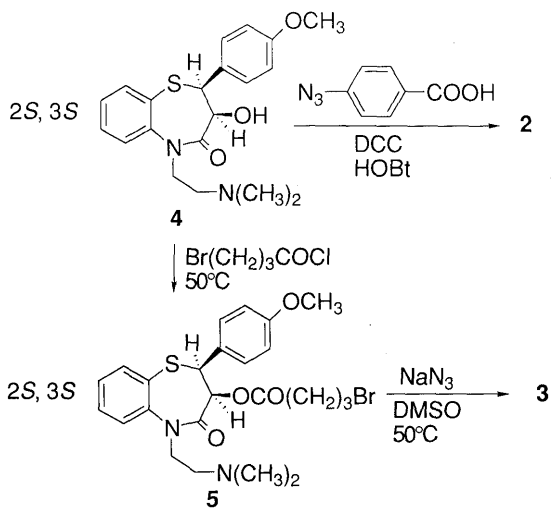

Chart 1

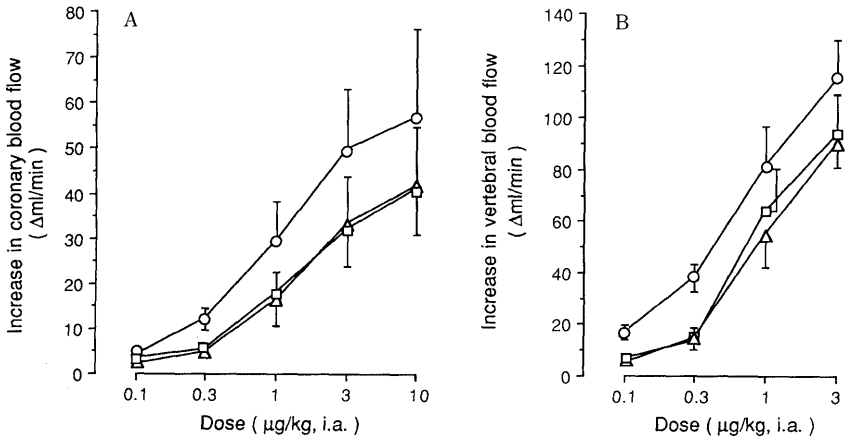

Fig. 2. Increasing Actions of Test Compounds on the Coronary (Panel A, Baseline: 38.2±8.4 ml/min) and Vertebral (Panel B, Baseline: 27.8±3.2 ml/min) Blood Flow in the Anesthetized Dog

Drugs were administered intraarterially. Symbols and vertical bars indicate means±S.E.M. of 4 or 5 experiments. ○, diltiazem; △, azidobenzoyl-diltiazem; □, azidobutyryldiltiazem.

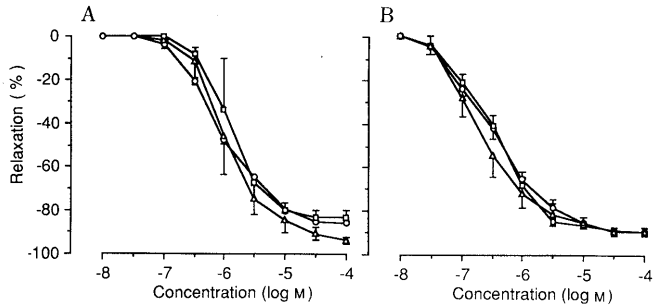

Fig. 3. Vasorelaxing Actions of Test Compounds on the Isolated and K^+-Depolarized Canine Coronary (Panel A) and Basilar (Panel B) Artery

Symbols and vertical bars indicate means ± S.E.M. of 3 or 4 experiments. ○, diltiazem; △, azidobenzoyl-diltiazem; □, azidobutyryl-diltiazem.

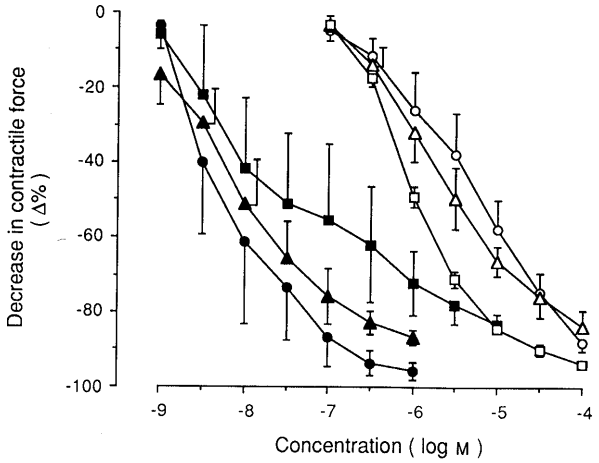

Fig. 4. Negative Inotropic Actions of Test Compounds in the Normal PSS (○, △, □) and K^+ Depolarizing PSS (●, ▲, ■) in the Isolated Guinea Pig Papillary Muscle

Symbols and vertical bars indicate means ± S.E.M. of 3 to 7 experiments. ○●, diltiazem; △▲, azidobenzoyl-diltiazem; □■, azidobutyryl-diltiazem.

(MC6621, Graphtec, Tokyo). The resting tension was adjusted to 0.8—1.3 g and the tissue was equilibrated for at least 1 h. Drugs were cumulatively administered at the tonic phase of K^+ contraction (40 mM).

Diltiazem exhibited concentration dependent relaxing action at concentrations of 10^{-7} M or more on the coronary artery and at concentrations of 3×10^{-8} M or more on the basilar artery (Fig. 3). Diltiazem relaxed both preparations by 90% at the concentration of 3×10^{-5} M.

Both azido compounds showed quite similar vasorelaxing actions to diltiazem in these preparations.

Negative Inotropic Action Ten male guinea pigs (Hartley strain, Shizuoka Laboratory Animal Center) weighing 296—396 g were killed by means of a blow on the head and the hearts were excised. The left ventricular papillary muscle was immediately isolated and suspended in an organ bath containing 10 ml of oxygenated PSS (95% O_2 and 5% CO_2, 30 °C). Isometric tension was measured and recorded as mentioned before. The resting tension was adjusted to approximately 1 g and contraction was induced by field electric stimulation of 1 Hz for 5—10 ms at supramaximal voltage (DPS-10, Dia Medical, Tokyo). Cumulative administration of the drug was started after the contraction became stable.

The negative inotropic action of the drug was also studied under K^+ depolarization. In this experiment, KCl (final concentration, 25 mM) was added after the contraction had become stable and then isoproterenol was added 10—20 min later to induce a Ca^{2+} dependent contraction. Drug administration was performed as mentioned above.

Negative inotropic actions of the test compounds in the normal PSS and in the depolarizing PSS are shown in Fig. 4. When compared in terms of EC_{50} values, the negative inotropic actions of azidobenzoyl-diltiazem and azidobutyryl-diltiazem were about two and about three times stronger than that of diltiazem in normal PSS, respectively. However, azidobenzoyl-diltiazem and azidobutyryl-diltiazem were about one half and about one-seventh as potent as diltiazem in the depolarizing solution, respectively.

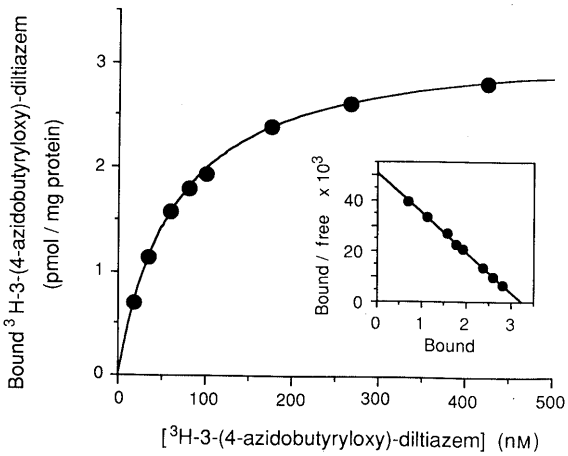

Fig. 5. Saturation Isotherm for [^{3}H]Azidobutyryl-diltiazem Specific Binding to Rat Skeletal Muscle Microsomes

Membranes were incubated with various concentrations of [^{3}H]azidobutyryl-diltiazem (10—500 nM) for 180 min at 0 °C. Symbols indicate means of five independent experiments, each done in duplicate. Inset: Scatchard analysis of the saturation experiments was used to determine the K_D and B_{max} values for [^{3}H]azidobutyryl-diltiazem.

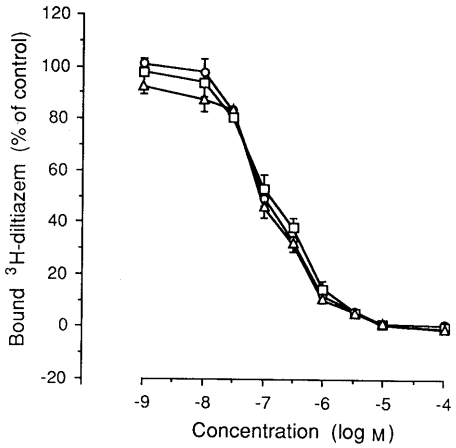

Fig. 6. Dose–Response Curves of the Effects of Diltiazem (○), Azidobenzoyl-diltiazem (△) and Azidobutyryl-diltiazem (□) on [^{3}H]-Diltiazem Binding to Rat Skeletal Muscle Microsomes

Symbols and vertical bars indicate means ± S.E.M. of 4 or 6 experiments.

Receptor Binding Assay in Skeletal Muscle Microsomes Skeletal muscle microsomes were prepared from 17 male rats weighing 250—410 g (SD strain, Charles River Japan) by the method of Glossmann *et al.*[11] The binding study was performed under the incubating condition of 2 °C and 3 h as described by Schwartz *et al.*[12]

As shown in Fig. 5, Scatchard analysis of [^{3}H]azidobutyryl-diltiazem binding indicated a dissociation constant (K_D) of 85.9 ± 12.9 nM and the maximal number of binding sites (B_{max}) of 3.6 ± 0.2 pmol/mg·protein in rat skeletal muscle microsomes. These values were in close agreement with the binding parameter of [^{3}H]diltiazem in the same preparation (data not shown).

We also studied [^{3}H]azidobenzoyl-diltiazem under the same conditions, but failed to identify the specific binding because of its nonspecific binding.

Nonlabeled diltiazem and its azido derivatives displaced the binding of [^{3}H]diltiazem to skeletal muscle microsomes (Fig. 6). There were no apparent differences among their inhibitory actions, and none of the Hill slopes for inhibition differed significantly from unity. Similar results were obtained from the experiment using skeletal muscle microsomes and [^{3}H]azidobutyryl-diltiazem as a ligand (data not shown).

Influence on [^{3}H]Nitrendipine Binding The effects of the test compounds on [^{3}H]nitrendipine binding to rat cerebral cortex synaptosomes (SD strain, 260—330 g, $N = 8$, Charles River Japan) were studied as described by Yamamura *et al.*[13] at 37 or 25 °C, for 60 min duration.

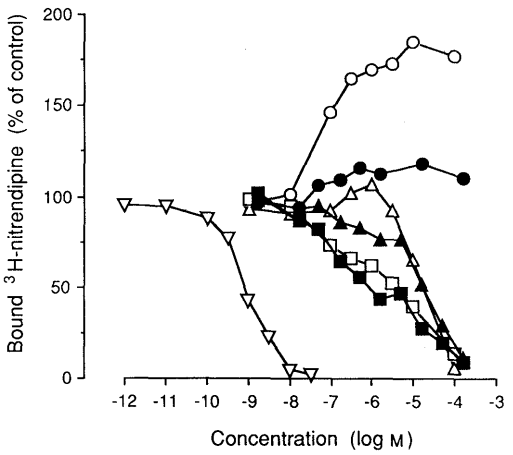

Fig. 7. Effects of Diltiazem and Azido Derivatives on [^{3}H]Nitrendipine Binding to the Rat Cerebral Cortex Synaptosomes at 37°C (○, △, □) and 25°C (●, ▲, ■)

The data represents means of 3 or 4 experiments, each done in duplicate. ○, ●, diltiazem; △▲, azidobenzoyl-diltiazem; □■, azidobutyryl-diltiazem; ▽, nitrendipine.

This study was performed in a dark room.

As shown in Fig. 7, diltiazem, at 37 °C, increased the [^{3}H]nitrendipine binding at concentrations of 10^{-7} M or more. This enhancement was the largest at the concentration of 10^{-5} M, and reached approximately 180% of the baseline value. At the same temperature, both azido derivatives showed inhibitory actions on [^{3}H]nitrendipine binding and they almost completely inhibited the binding at the highest concentration of 10^{-4} M. At the temperature of 25 °C, diltiazem still slightly enhanced [^{3}H]nitrendipine binding, but azido derivatives exhibited similar actions to those observed at 37 °C.

Discussion

As mentioned before, we synthesized azidobenzoyl-diltiazem and azidobutyryl-diltiazem and studied their pharmacological and radioligand binding properties with the aim of employing them as tools for investigation by photoaffinity labeling of the binding site of 1,5-benzothiazepine Ca antagonists on the voltage dependent Ca channel. The results were as follows.

1) Both azido compounds demonstrated similar increasing actions to diltiazem, though slightly less potent, on dog coronary and vertebral blood flow when administered by intraarterial injection.

2) Both azido compounds showed quite similar vasorelaxing actions to diltiazem in the isolated and K^+-depolarized canine coronary and basilar arteries.

3) Although their negative inotropic actions were stronger than that of diltiazem in the normal PSS, they were less potent negative inotropic agents than diltiazem when studied in the K^+ depolarizing PSS, in which the cardiac voltage-dependent Ca channel was activated.

4) Both azido compounds shared a common binding site with diltiazem in skeletal muscle microsomes. Although the K_D and B_{max} of [^{3}H]azidobenzoyl-diltiazem could not be estimated because of its nonspecific binding, [^{3}H]azidobutyryl-diltiazem demonstrated similar properties to diltiazem.

Thus, our azido compounds were considered to have pharmacologically similar properties to diltiazem in the vascular smooth muscle and to share common binding sites with diltiazem in the skeletal muscle microsomes.

But they exhibited distinctive properties in cardiac muscle. Moreover, diltiazem and its azido derivatives affected [^{3}H]nitrendipine binding to rat cerebral cortex synaptosomes in a different fashion, suggesting distinct allosteric effects on the nitrendipine binding. These findings suggest a difference among the voltage dependent Ca channels in various tissues as well as among 1,5-benzothiazepine derivatives. Schwartz *et al.* reported the presence of an organ difference in the α_1 subunit of the voltage-dependent Ca channel which Ca antagonists bind to.[10] Our results could be explained in terms of such a subtype difference of α_1 subunit.

In conclusion, our azido compounds were considered to share a common binding site on the voltage-dependent Ca channel with diltiazem, because they demonstrated basically similar pharmacological radioligand binding characteristics to diltiazem.

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