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High-affinity binding of [^{3}H]DTZ323 to the diltiazem-binding site of L-type Ca^{2+} channels

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Abstract

D-*cis*-[*N*-Methyl-^{3}H]-3-(acetyloxy)-5-[2-[[2-(3,4-dimethoxyphenyl)ethyl]-methylamino]ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepine-4(5*H*)-one ([^{3}H]DTZ323), a novel 1,5-benzothiazepine radioligand, was characterized in a ligand-receptor binding study. Specific binding of [^{3}H]DTZ323 to rabbit skeletal muscle T-tubule membranes was saturable and reversible. Scatchard analysis indicated a single binding site with a K_d value of 1.4 and 1.8 nM at 25 and 37 °C, respectively. DTZ323 and diltiazem derivatives inhibited specific [^{3}H]DTZ323 binding with a rank order of DTZ323>DTZ417 (quaternary ammonium derivative of DTZ323)>diltiazem>L-*cis*-DTZ323. The affinity of DTZ323 was 51 times higher than that of diltiazem. [^{3}H]DTZ323 binding was also completely inhibited by verapamil and tetrandrine, thus revealing the unique nature of the diltiazem-binding site. Specific [^{3}H]DTZ323 binding to crude guinea pig ventricular membranes was inhibited by diltiazem, DTZ323 and its derivatives with IC_{50} values close to those previously reported for the blockade of L-type Ca^{2+} channel currents. These results indicate that [^{3}H]DTZ323 is a potent and selective radioligand for the diltiazem-binding site of L-type Ca^{2+} channels.

Keywords: Ca^{2+} channel, L-type; Diltiazem; Ca^{2+} channel antagonist; Dihydropyridine receptor; Ca^{2+}; Radioligand binding

1. Introduction

Ca^{2+} channel antagonists such as nifedipine, verapamil and diltiazem belong to three representative chemical classes: 1,4-dyhydropyridines, phenylalkylamines and 1,5-benzothiazepines. Each class of Ca^{2+} channel antagonists exhibits distinct characteristics in their tissue selectivity (Triggle, 1991). They are known to bind to distinct binding sites within the α_1 subunit of the L-type Ca^{2+} channel and to have a reciprocal allosteric interaction (Adachi-Akahane and Nagao, 2000; Mitterdorfer et al., 1998). Ca^{2+} channel antagonists have also been used as specific probes for the pharmacological and structural characterization of the L-type Ca^{2+} channel (Spedding and Paoletti, 1992).

Among the Ca^{2+} channel antagonists, diltiazem is clinically used for angina pectoris, hypertension and supraventricular tachycardia (Adachi-Akahane and Nagao, 2000). The unique tissue selectivity of diltiazem is explained by the voltage-dependence of its interaction with L-type Ca^{2+} channels (Okuyama et al., 1994; Adachi-Akahane and Nagao, 2000). However, the molecular mechanisms underlying the voltage-dependent modulation of the gating properties of Ca^{2+} channels by diltiazem have not been clarified because of the low affinity of radioligands for the diltiazem-binding site of the Ca^{2+} channel. In receptor binding studies, several radioligands, such as [^{3}H]diltiazem, [^{3}H]azidobutyryl-diltiazem and [^{3}H]clentiazem, have been used to characterize 1,5-benzothiazepine-binding sites within L-type Ca^{2+} channels in membranes isolated from skeletal muscle or cardiac muscle (Glossmann et al., 1983; Balwierczak et al., 1987; Naito et al., 1989; Narita et al., 1990; Zobrist and Mecca, 1990). However, the binding affinities of those radioligands for the 1,5-benzothiazepine-binding sites were lower than those of radiolabeled dihydropyridines or phenylalkylamines. For this reason, the radioligand-receptor interaction at the 1,5-benzothiazepine receptor site has not been investigated as extensively as those sites for phenylalkylamines or dihydropyridines (Catterall and Striessnig, 1992). Thus, high-affinity radioligands for the 1,5-benzothiazepine site may be a useful tool for further investigation of the 1,5-benzothiazepine-binding site

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and other binding sites that allosterically interact with 1,5-benzothiazepine-binding sites within the L-type Ca^{2+} channel.

The 3,4-dimethoxyphenyl derivative of diltiazem, 3-(acetyloxy)-5-[2-[[2-(3,4-dimethoxyphenyl)ethyl]-methylamino]ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepine-4(5*H*)-one (DTZ323) (Fig. 1), is a D-*cis*-benzothiazepine derivative and has been characterized in our laboratory (Hagiwara et al., 1997; Kurokawa et al., 1997a,b). We showed that it binds selectively to 1,5-benzothiazepine sites with an affinity (K_i = 6.6 nM) higher than that of diltiazem (K_i = 314 nM) or clentiazem (K_i = 61 nM) in [^{3}H]diltiazem-binding experiments (Hagiwara et al., 1997) and that it blocks L-type Ca^{2+} channel currents selectively in single guinea pig ventricular myocytes (Kurokawa et al., 1997a). DTZ323 blocked Ca^{2+} channel currents in a voltage-dependent manner (Kurokawa et al., 1997a). DTZ323 and its membrane-impermeable quaternary ammonium derivative (DTZ417) have been reported to block Ca^{2+} channel currents preferentially from the extracellular side of the membrane, suggesting that the specific binding site for DTZ323 is accessible from the extracellular side (Kurokawa et al., 1997b). These functional and binding studies showed that DTZ323 is the most potent ligand for the diltiazem-binding site studied so far. Therefore, in the present study, we further characterized the binding properties of DTZ323 by using [^{3}H]DTZ323 (Fig. 1). First, a detailed characterization was performed with respect to the kinetic and equilibrium binding properties of specific [^{3}H]DTZ323 binding to rabbit skeletal muscle L-type Ca^{2+} channels. Next, the pharmacological profile of the [^{3}H]DTZ323-binding site was studied. The affinity of DTZ323, DTZ417, D-*cis*-DTZ323 and verapamil for [^{3}H]DTZ323-binding sites in cardiac L-type Ca^{2+} channels was also estimated in comparison with that of diltiazem in crude membranes from guinea pig ventricles.

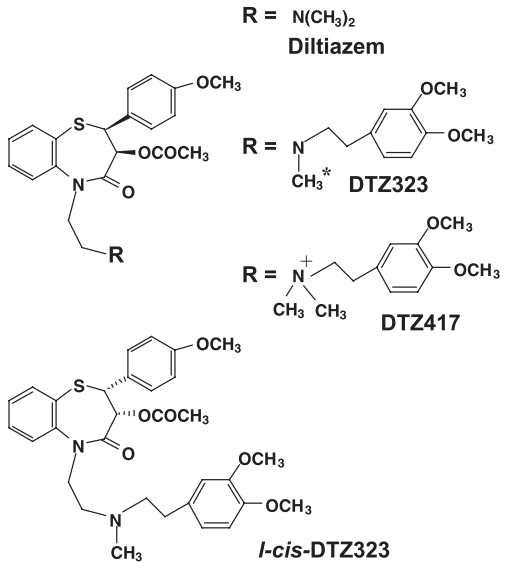

Fig. 1. Chemical structures of DTZ323 derivatives and diltiazem. The asterisk indicates the position of radiolabeling.

2. Materials and methods

2.1. Membrane preparation

All procedures for animal use were carried out in accordance with *Guidelines for Animal Experimentation* (Japanese Association for Laboratory Animal Science, 1987) and the *Guidelines of animal experiments in University of Tokyo*.

Membranes were prepared from rabbit skeletal muscle and guinea pig ventricular myocardium according to Hagiwara et al. (1997). Briefly, back muscles were removed from New Zealand white rabbits (male, 1–1.5 kg). Ventricles were quickly excised from Hartley guinea pigs (male, 250 g). The tissue was minced with scissors and homogenized with a glass-Teflon homogenizer, followed by a Polytron (settings 7–8, 10 s × 3 for skeletal muscles and 15 s × 2 for ventricular muscles) in five to seven volumes of ice-cold buffer A that contained $NaHCO_3$, 20 mM; phenylmethylsulfonyl fluoride, 0.2 mM; iodo-acetamide, 1 mM; pepstatin A, 1 μM. After filtration through two layers of cheesecloth and centrifugation at 1500 × *g* for 15 min, the supernatant was filtered again and centrifuged at 45,000 × *g* for 20 min. The pellet was resuspended in ice-cold buffer B (Tris–HCl, 50 mM; phenylmethylsulfonyl fluoride, 0.2 mM; iodo-acetamide, 1 mM; pepstatin A, 1 μM; pH 7.4 at 4 °C), centrifuged and washed with the same buffer again. The pellet was finally resuspended in buffer B and stored at −70 °C. All procedures were carried out at 4 °C. Protein concentration was measured according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

2.2. Binding assay

DTZ323 was tritium labeled (Amersham-Pharmacia Biotech, Buckinghamshire, UK) at the position indicated by the asterisk in Fig. 1. The specific activity of radiolabeled DTZ323 was 84 Ci/mmol and its purity was 98.4% in stock solution. Radioligand binding studies with [^{3}H]DTZ323 were carried out at 2, 25 and 37 °C in 50 mM Tris–HCl (pH 7.4 at respective incubation temperature), as described previously (Hagiwara et al., 1997). Protein concentration and total assay volume were 0.0375 mg/assay and 0.5 ml, respectively.

In association kinetic experiments, membranes were incubated with [^{3}H]DTZ323 (0.3, 1.2 and 3.3 nM) for various time periods. The dissociation reaction was initiated by addition of 5 μM DTZ323 to membranes that had been incubated with 1.2 nM [^{3}H]DTZ323 to reach equilibrium. Nonspecific binding was measured in the presence of 1 μM DTZ323.

In saturation experiments, membranes were incubated with [^{3}H]DTZ323 over a concentration range of 0.2–63 nM for 90 min at 25 °C or for 60 min at 37 °C. Nonspecific binding was determined by the addition of DTZ323 at 1 μM.

In the competition experiments, membranes were incubated with 1.2 or 1.5 nM of [^{3}H]DTZ323 at 25 °C and nonspecific binding was determined with DTZ323 at 1 μM. When the Ca^{2+} dependence of the binding was tested, 4 nM [^{3}H]DTZ323 was used for the competition experiments at 37 °C in the presence or absence of Ca^{2+} and the nonspecific binding was determined by the addition of 10 μM DTZ323.

At the end of the incubation period, samples were diluted with 5 ml of ice-cold washing buffer (50 mM Tris–HCl, pH 7.4) and immediately filtered through GF/C filters presoaked in 0.5% polyethyleneimine and 0.1% bovine serum albumin to reduce nonspecific binding to filters. Filters were washed three times with 5 ml of ice-cold buffer. A Brandel cell harvester (Biomedical Research and Development Laboratories) was used for the filtration procedure. Radioactivity on filters was measured by liquid scintillation counting. All experiments were performed in duplicate and results are presented as means ± S.E.

2.3. Data analysis

Data were analyzed with nonlinear least-squares programs. The SP123 program by H. Ono (Nagoya City University) was used for Scatchard analysis of saturation binding data and pseudo-Scatchard analysis of inhibition binding data to obtain values for K_d and B_{max}, as reported previously (Hagiwara et al., 1997). In the case of pseudo-Scatchard analysis, specific bound and free ligand concentrations were calculated according to the following equations:

Specific bound (nM)=(measured total count (dpm) − measured nonspecific count (dpm)) × ([^{3}H]DTZ323 (nM) + DTZ323 (nM))/[^{3}H]DTZ323 added (dpm).

Free ligand concentration (nM)=([^{3}H]DTZ323 (nM) + DTZ323 (nM)) × ([^{3}H]DTZ323 added (dpm) − measured nonspecific count (dpm))/[^{3}H]DTZ323 added (dpm) − spe-specific bound (nM).

The LBS program (see Hagiwara et al., 1997) was used to calculate IC_{50} values and the slope factor from the inhibition binding data. Values of the inhibition constant (K_i) were calculated from IC_{50} values using the following equation: $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of the radioligand (Cheng and Prusoff, 1973).

2.4. Drugs

[^{3}H]DTZ323 (84 Ci/mmol) was tritiated by Amersham-Pharmacia Biotech. DTZ323, DTZ417, L-*cis*-DTZ323 and diltiazem were kindly supplied by Tanabe Seiyaku (Saitama, Japan). Tetrandrine was kindly supplied by Tsumura (Tokyo, Japan). Nicardipine, pepstatin A, phenylmethylsulfonyl fluoride and iodo-acetamide were purchased from Sigma (St. Louis, MO, USA). (±)-Verapamil (Verapamil) was purchased from Nacalai Tesque (Kyoto, Japan).

3. Results

3.1. Kinetic characteristics of [^{3}H]DTZ323 binding

First, we examined the rate of association and dissociation of the specific binding of [^{3}H]DTZ323 to rabbit skeletal muscle membranes at 25 °C. The dissociation rate constant (K_{-1}) was estimated to be 0.032 ± 0.002 min^{-1} ($n = 3$), being the slope of the correlation between $\ln(B_0/B_t)$ and t, where B_0 and B_t denote the concentration of bound ligands at time 0 and t of dissociation, respectively. The association rate constant (K_{+1}) was calculated to be 0.022 ± 0.009 nM^{-1} min^{-1} ($n = 3$) from K_{-1} and K_{obs}, according to the equation: $K_{+1} = (K_{obs} - K_{-1})/[L]$, where K_{obs} ($K_{obs} = 0.057 \pm 0.008$ min^{-1}, $n = 3$) was calculated as the slope of the correlation between $\ln(B_{eq}/(B_{eq} - B_t))$ and t, with B_{eq} and [L] representing the concentrations of bound ligand at equilibrium and the concentration of ligand, respectively. The K_d value was calculated to be 1.5 nM according to the equation: $K_d = K_{-1}/K_{+1}$.

Next, we performed another association binding experiment to obtain the K_d value for [^{3}H]DTZ323 binding at 25 °C. K_{obs}, K_{+1} and K_{-1} were determined by changing the concentration of [^{3}H]DTZ323 ([L]) in accordance with the following equation: $K_{obs} = K_{-1} + K_{+1}[L]$. Typical data are shown in Fig. 2. Fig. 2A shows the time course of the [^{3}H]DTZ323 association reaction at three concentrations of 0.3, 1.2 and 3.3 nM. The association reaction of [^{3}H]DTZ323 reached a maximum within 1 h and the binding remained constant for at least another 90 min. Fig. 2B shows plots of $\ln(B_{eq}/(B_{eq} - B_t))$ as a function of time (t). K_{obs} values, obtained from this equation for [^{3}H]DTZ323 binding at 0.3, 1.2 and 3.3 nM, were 0.045 ± 0.001, 0.068 ± 0.002 and 0.105 ± 0.004 min^{-1}, respectively ($n = 3$). Fig. 2C shows K_{obs} plotted as a function of [^{3}H]DTZ323 concentration. K_{+1} calculated from the slope was 0.021 ± 0.001 nM^{-1} min^{-1} ($n = 3$). K_{-1} calculated as the intercept of the ordinate was 0.041 ± 0.003 min^{-1} ($n = 3$). The K_d value was calculated to be 1.97 ± 0.23 nM ($n = 3$), according to the equation $K_d = K_{-1}/K_{+1}$. The K_d value obtained here was consistent with that obtained in the kinetic experiments (1.5 nM) and saturation experiments (1.4 nM) described in Section 2.

[^{3}H]DTZ323 binding at 37 °C was also reversible and equilibrium binding was established within 10 min; at 2 °C, the time course was slower and the association reaction was still submaximal even at 6 h.

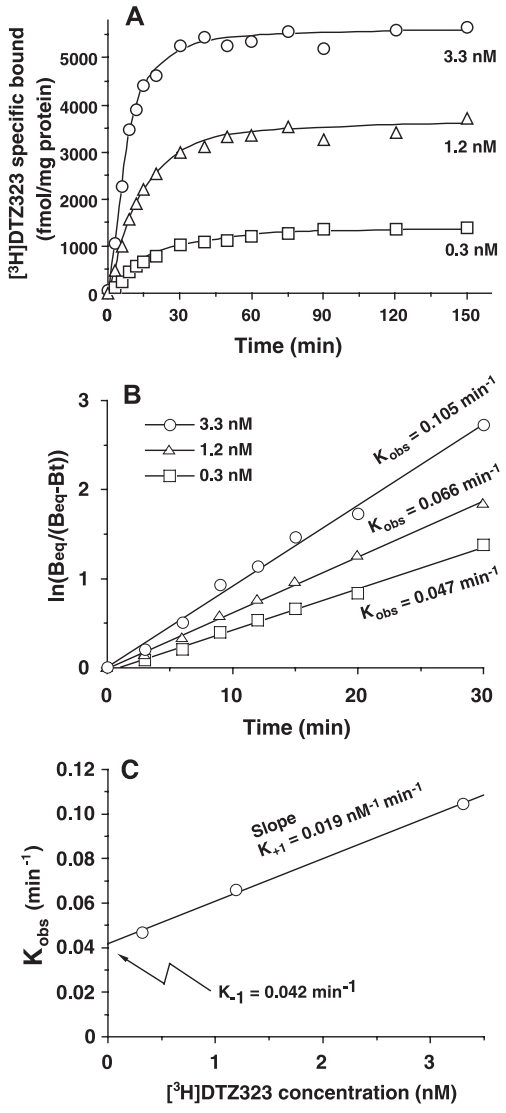

Fig. 2. Association time course and kinetic constants of [^{3}H]DTZ323 binding to rabbit skeletal muscle membrane at 25 °C. Membranes were incubated with different concentrations of [^{3}H]DTZ323 for various time periods. Nonspecific binding was determined in the presence of 1 μM DTZ323. (A) Association time course. (B) $\mathrm{Ln}(B_e/B_e - B_t)$ vs. t plot. K_{obs} was obtained as a slope. (C) K_{obs} vs. [^{3}H]DTZ323 concentration plot. Kinetic constants of [^{3}H]DTZ323 binding obtained from the K_{obs} vs. concentration of [^{3}H]DTZ323 plot. K_{+1} and K_{-1} were obtained from the slope and the ordinate, respectively. Calculated K_d value was 2.2 nM.

3.2. Binding isotherm and Scatchard plot of [^{3}H]DTZ323 binding

Fig. 3 shows a typical binding isotherm and Scatchard plot of data from saturation binding experiments at 25 °C with rabbit skeletal muscle membranes. Scatchard analysis of saturation isotherms indicated that [^{3}H]DTZ323 bound to a single high-affinity class of binding site, with a Hill coefficient close to unity. The K_d value, B_{max} and slope factor were 1.4 ± 0.1 nM, 7915 ± 374 fmol/mg protein and 1.05 ± 0.07, respectively. The K_d value obtained from the Scatchard plot was consistent with the value calculated according to K_{-1}/K_{+1} in Section 2. The nonspecific binding, obtained in the presence of 1 μM DTZ323, increased linearly with increasing concentrations of [^{3}H]DTZ323. At 1.7 ± 0.2 nM, a concentration close to the K_d value for specific [^{3}H]DTZ323 binding to membranes, nonspecific binding was 4.6 ± 0.4% of the total binding (n = 3). Specific binding increased linearly with the amount of membrane protein over a concentration range of 0.01–0.1 mg protein/assay.

Equilibrium binding experiments were also carried out at 37 °C. K_d value, B_{max} and slope factor were 1.8 ± 0.2 nM, 4985 ± 144 fmol/mg protein and 1.06 ± 0.05, respectively. Nonspecific binding, obtained in the presence of 1 μM DTZ323 at 37 °C, was 10.5 ± 0.2% (n = 4) of the total binding with [^{3}H]DTZ323 at 1.86 ± 0.12 nM, which is close to the K_d value.

3.3. Inhibition of [^{3}H]DTZ323 binding by DTZ323 derivatives, diltiazem, verapamil and tetrandrine at 25 °C

At 25 °C, DTZ323, DTZ417, L-*cis*-DTZ323 and diltiazem completely inhibited [^{3}H]DTZ323 binding to the skeletal muscle membranes in a concentration-dependent manner with a slope factor close to unity, which indicates that [^{3}H]DTZ323 binding was competitively inhibited by diltiazem and its structurally related derivatives. The rank order of binding affinity was DTZ323>DTZ417>diltiazem>L-*cis*-DTZ323. Table 1 summarizes the IC_{50} values, K_i values and slope factors for the inhibition of [^{3}H]DTZ323 binding to rabbit skeletal muscle membranes at 25 °C (shown in Fig. 4). The K_i value of DTZ323 was 51 times

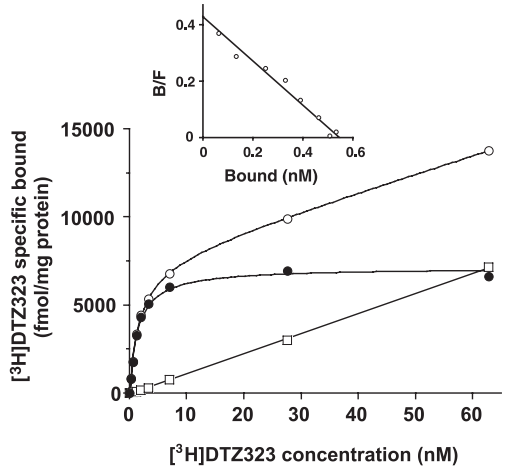

Fig. 3. Equilibrium binding isotherm and Scatchard plot of [^{3}H]DTZ323 binding to rabbit skeletal muscle membranes at 25 °C. Total binding of [^{3}H]DTZ323 was measured over the concentration range of 0.2–63 nM (○). Nonspecific binding was measured over the same concentration range in the presence of unlabeled 1 μM DTZ323 (□). Specific binding was determined as the difference between total binding and nonspecific binding (●). The experiment was performed in duplicate. Inset, Scatchard plot. Calculated K_d and B_{max} values were 1.3 nM and 7204 fmol/mg protein, respectively.

Table 1
Inhibition of [^{3}H]DTZ323 binding to rabbit skeletal muscle membrane at 25 °C

Drug	IC_{50} (nM)	K_i (nM)	Slope factor
DTZ323	6.5 ± 0.2	3.1 ± 0.1	1.07 ± 0.01
DTZ417	86 ± 2	41 ± 1	1.16 ± 0.01
L-*cis*-DTZ323	597 ± 10	282 ± 15	1.04 ± 0.01
Diltiazem	337 ± 11	159 ± 9	0.96 ± 0.03
Verapamil	86 ± 7	–	0.99 ± 0.03
Tetrandrine	229 ± 12	113 ± 6	0.95 ± 0.04

Membranes were incubated at 25 °C for 90 min with 1.5 nM [^{3}H]DTZ323 in the presence of various concentrations of DTZ323, DTZ417, L-*cis*-DTZ323, diltiazem, verapamil or tetrandrine. Nonspecific binding was measured in the presence of 1 μM DTZ323. Each value represents the mean ± S.E. of four experiments (DTZ323, DTZ417, L-*cis*-DTZ323, diltiazem and tetrandrine) or three experiments (verapamil).

smaller than that of diltiazem. Stereospecificity for DTZ323 (D-*cis*) over its L-*cis* isomer was demonstrated by differences in K_i values of approximately two orders of magnitude.

Verapamil has been reported to inhibit [^{3}H]diltiazem binding in a noncompetitive manner through a negative allosteric interaction, although verapamil apparently mimics competitive interaction (Garcia et al., 1986; Balwierczak et al., 1987). In this study, verapamil also completely inhibited [^{3}H]DTZ323 binding with a slope factor close to unity.

Tetrandrine is an alkaloid known to block the L-type Ca^{2+} channel via an interaction with the diltiazem-binding site (King et al., 1988). Tetrandrine inhibited [^{3}H]DTZ323 binding with a slope factor close to unity at 25 °C.

3.4. Influence of Ca^{2+} on the affinity of Ca^{2+} channel antagonists for the [^{3}H]DTZ323-binding site

Among Ca^{2+} channel antagonist ligands, dihydropyridines and benzothiazepines are known to exert a reciprocal allosteric interaction (Adachi-Akahane and Nagao, 2000; Mitterdorfer et al., 1998). Dihydropyridine ligands have been reported to enhance [^{3}H]diltiazem binding to skeletal muscle L-type Ca^{2+} channels at 30 °C and to inhibit it at 2 °C (Glossmann et al., 1983). It is reported that diltiazem augments [^{3}H]dihydropyiridine binding at 37 °C, inhibits it at 2 °C, but has no effect at 25 °C as a result of changes in the association and dissociation kinetics of the ligand (Ikeda et al., 1991). These allosteric interactions reflect the functional interaction between the two drug-binding sites in cardiac L-type Ca^{2+} channels (Kanda et al., 1998). In order to elucidate the dependence of the diltiazem-binding site on Ca^{2+} with respect to the positive/negative allosteric interaction with the dihydropyridine-binding site, we also examined the effect of nicardipine on [^{3}H]DTZ323 binding in the absence or the presence of 1 mM Ca^{2+} at 37 °C and compared the manner of modulation with that of DTZ323, diltiazem, tetrandrine or verapamil.

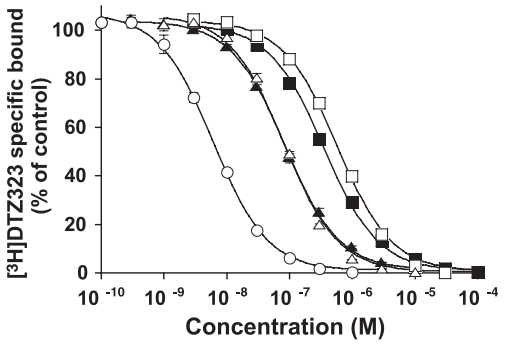

Fig. 4. Effects of DTZ323 stereoisomers, diltiazem and verapamil on [^{3}H]DTZ323 binding to rabbit skeletal muscle membranes. Membranes were incubated at 25 °C for 90 min with 1.5 nM [^{3}H]DTZ323 in the presence of various concentrations of DTZ323 (○), DTZ417 (△), L-*cis*-DTZ323 (□), diltiazem (■) and verapamil (▲). Nonspecific binding was measured in the presence of 1 μM DTZ323. Each point represents the mean ± S.E. of four experiments.

According to the simplified allosteric models described by Ehlert (1988) and Tomlinson and Hnatowich (1988), inhibition of the specific binding of a radioligand by an allosteric modulator is likely to be observed as apparent competitive inhibition when the magnitude of the negative heterotropic cooperativity is very large and the concentration of the radioligand is much smaller than its K_d value. To examine the allosteric nature of the interaction in the competition binding studies, [^{3}H]DTZ323 was used at a concentration of 4 nM, a concentration higher than the calculated K_d value of 1.5 nM. K_i values for each Ca^{2+} channel antagonist are summarized in Table 2.

In the absence of 1 mM Ca^{2+}, nicardipine completely inhibited [^{3}H]DTZ323 binding to the membranes at 37 °C (Fig. 5A). In contrast, in the presence of 1 mM Ca^{2+}, nicardipine partially inhibited [^{3}H]DTZ323 binding (maximal inhibition = 90 ± 1%, n = 3, Fig. 5B). Ca^{2+} shifted the inhibition curve for nicardipine to the left (Fig. 5 and Table 2). DTZ323, diltiazem, tetrandrine, and verapamil completely

Table 2
Inhibition of [^{3}H]DTZ323 binding to rabbit skeletal muscle membrane at 37 °C in the absence or presence of 1 mM $CaCl_2$

Drug	IC_{50} (nM)	K_i (nM)	Slope factor
DTZ323, Ca^{2+} (−)	19 ± 0.2	14 ± 0.1	1.05 ± 0.02
DTZ323, Ca^{2+} (+)	47 ± 1	44 ± 1	1.15 ± 0.03
Diltiazem, Ca^{2+} (−)	1030 ± 63	760 ± 46	1.04 ± 0.01
Diltiazem, Ca^{2+} (+)	2464 ± 12	2300 ± 11	1.14 ± 0.01
Verapamil, Ca^{2+} (−)	189 ± 9	–	1.06 ± 0.01
Verapamil, Ca^{2+} (+)	490 ± 58	–	1.06 ± 0.04
Tetrandrine, Ca^{2+} (−)	83 ± 9	61 ± 6	1.05 ± 0.07
Tetrandrine, Ca^{2+} (+)	168 ± 2	157 ± 1	1.21 ± 0.15
Nicardipine, Ca^{2+} (−)	17 ± 1	–	1.16 ± 0.01
Nicardipine, Ca^{2+} (+) (maximal inhibition = 90 ± 1%)	8.4 ± 0.3[a]	–	1.23 ± 0.04[a]

Membranes were incubated at 37 °C for 60 min with 4 nM [^{3}H]DTZ323 and various concentrations of DTZ323, diltiazem, verapamil, tetrandrine and nicardipine in the absence or presence of 1 mM Ca^{2+}. Nonspecific binding was measured in the presence of 10 μM DTZ323. Each value represents the mean ± S.E. of three experiments.

[a] The value for nicardipine in the presence of Ca^{2+} was calculated from the concentration that produces 50% inhibition of the maximal inhibition by nicardipine.

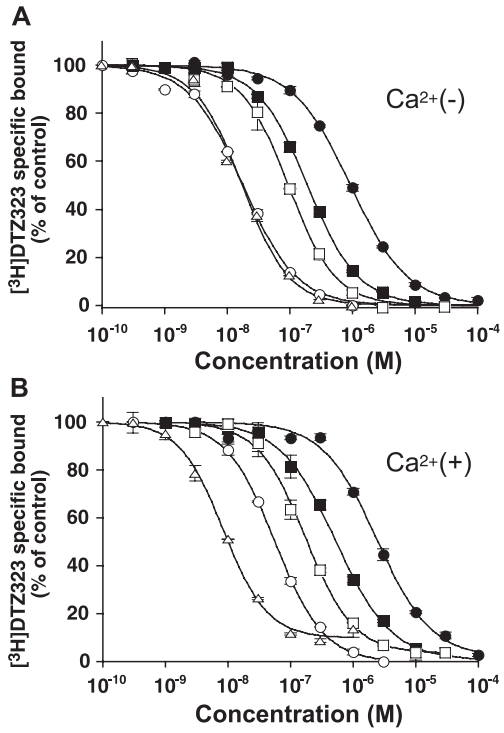

Fig. 5. Effects of Ca^{2+} on the competition of [^{3}H]DTZ323 binding by Ca^{2+} channel antagonists at 37 °C. Rabbit skeletal muscle membranes were incubated at 37 °C for 60 min with 4 nM [^{3}H]DTZ323 in the presence of various concentrations of nicardipine (△), DTZ323 (○), diltiazem (●), tetrandrine (□) and verapamil (■) in the absence (A) or in the presence (B) of 1 mM $CaCl_2$. Nonspecific binding was measured in the presence of 10 μM DTZ323. Each point represents the mean ± S.E. of three experiments.

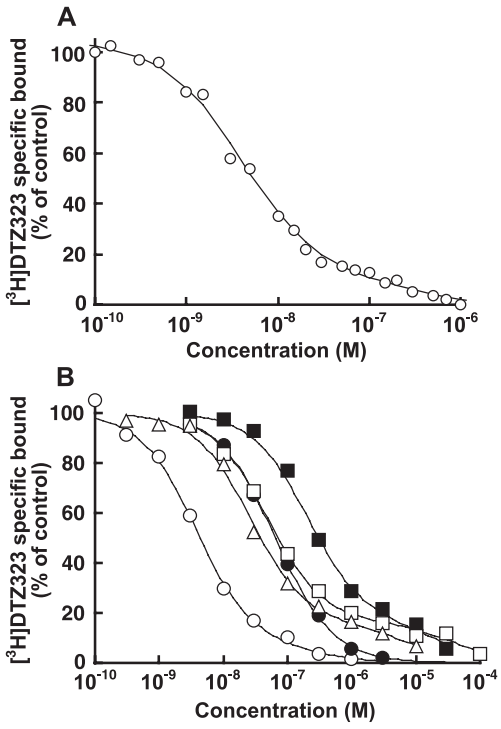

Fig. 6. Inhibition of [^{3}H]DTZ323 binding to cardiac membranes by DTZ323. Crude membranes from guinea pig ventricular muscles were incubated at 25 °C for 90 min with 1.5 nM [^{3}H]DTZ323 in the presence of various concentrations of DTZ323 (○), DTZ417 (●), diltiazem (□), L-*cis*-DTZ323 (■), and verapamil (△). Nonspecific binding was measured in the presence of 1 μM DTZ323. (A) Calculated IC_{50} values for specific [^{3}H]DTZ323 binding associated with high-affinity site (90%) and low-affinity site (10%) were 4.0 and 430 nM, respectively.

inhibited the binding of [^{3}H]DTZ323 to membranes both in the absence and in the presence of 1 mM Ca^{2+}. In contrast to the results obtained with nicardipine, the inhibition curves for DTZ323, diltiazem, tetrandrine or verapamil were shifted to the right in the presence of 1 mM Ca^{2+} (Fig. 5 and Table 2).

Pseudo-Scatchard analysis of the data for inhibition of 4 nM [^{3}H]DTZ323 binding to the membranes by unlabeled DTZ323, measured in the absence of Ca^{2+}, revealed a single class of binding site with K_d and B_{max} values of 12.5 ± 0.8 nM and 7885 ± 383 fmol/mg protein, respectively (n = 3). In contrast, in the presence of Ca^{2+} at 1 mM, K_d and B_{max} values were calculated to be 45.1 ± 1.8 nM and 7400 ± 349 fmol/mg protein, respectively (n = 3). The dissociation constant of [^{3}H]DTZ323 in the presence of 1 mM Ca^{2+} was significantly greater than that measured in the absence of Ca^{2+} (P < 0.0001 by Student's t-test).

3.5. Effect of DTZ323 derivatives, diltiazem, and verapamil on [^{3}H]DTZ323 specific binding to guinea pig ventricular membranes at 25 °C

We further estimated the affinity of DTZ323, DTZ417, L-*cis*-DTZ323, diltiazem and verapamil binding to cardiac L-type Ca^{2+} channels by using [^{3}H]DTZ323 and crude membranes from guinea pig ventricular muscles at 25 °C. DTZ323 exhibited biphasic inhibition of [^{3}H]DTZ323 binding, which indicates the presence of high- and low-affinity binding sites for [^{3}H]DTZ323 in cardiac membranes (Fig. 6A and Table 3). At a radioligand concentration of 1.5 nM, close to the K_d value obtained in skeletal muscle membranes, 84% of the total bound [^{3}H]DTZ323 was associated with the high-affinity site. The high-affinity site (80%) and the low-affinity site (20%) were also observed in the same

Table 3
Inhibition of [^{3}H]DTZ323 binding to guinea pig cardiac membranes at 25 °C

Drug	$IC_{50(H)}$ (nM)	R_H (%)	$IC_{50(L)}$ (μM)	R_L (%)
DTZ323	2.9 ± 0.4	84 ± 4	0.19 ± 0.09	16 ± 4
DTZ417	57 ± 8	92 ± 3	45 ± 41	8 ± 3
L-*cis*-DTZ323	211 ± 4	81 ± 2	15 ± 3	19 ± 2
Diltiazem	63 ± 6	84 ± 1	56 ± 8	16 ± 1
Verapamil	27 ± 2	83 ± 1	12 ± 5	17 ± 1

Membranes were incubated at 25 °C for 90 min with 1.5 nM [^{3}H]DTZ323 in the presence of various concentrations of DTZ323, DTZ417, L-*cis*-DTZ323, diltiazem or verapamil. Nonspecific binding was measured in the presence of 1 μM DTZ323. R_H and R_L represent the percentage of the specific binding associated with high- and low-affinity sites at the radioligand concentration, respectively. $IC_{50(H)}$ and $IC_{50(L)}$ indicate IC_{50} values for high- and low-affinity sites, respectively. Each value represents the mean ± S.E. of four experiments.

preparation in the competition experiments with [^{3}H]diltiazem (30 nM, data not shown). DTZ417, L-*cis*-DTZ323, diltiazem, and verapamil also exhibited biphasic inhibition of [^{3}H]DTZ323 binding (Fig. 6B and Table 3). At high-affinity binding sites in cardiac membranes, the potency for the inhibition of [^{3}H]DTZ323 binding by DTZ323 derivatives was similar to that obtained in rabbit skeletal muscle L-type Ca^{2+} channels, although the IC_{50} value for diltiazem was somewhat smaller in ventricular membranes than in skeletal muscle membranes. Stereospecificity of binding was observed for D-*cis* DTZ323 and its L-*cis* stereoisomer. The rank order of the binding affinity of 1,5-benzothiazepine derivatives to the high-affinity binding site for [^{3}H]DTZ323 was DTZ323>verapamil>DTZ417>diltiazem>L-*cis*-DTZ323, which was similar to what was observed in skeletal muscle membranes. In contrast, the rank order for the low-affinity [^{3}H]DTZ323-binding sites was DTZ323 ≫ verapamil>L-*cis*-DTZ323>DTZ417>diltiazem. DTZ417, a permanently charged derivative of DTZ323, inhibited the high-affinity binding site almost exclusively, which might be due to the membrane impermeability of this compound.

4. Discussion

Ligand-receptor binding experiments with radiolabeled DTZ323 were carried out to characterize the specific binding of DTZ323 to L-type Ca^{2+} channels in terms of kinetic and equilibrium properties of the interaction between DTZ323 and the 1,5-benzothiazepine receptor site.

In rabbit skeletal muscle membranes, kinetic experiments indicated that the specific binding of [^{3}H]DTZ323 to L-type Ca^{2+} channels was saturable and reversible. Scatchard analysis of saturation isotherms indicated that [^{3}H]DTZ323 bound to a single high-affinity class of binding site at 25 and 37 °C. Equivalent K_d values were obtained from the kinetic parameters and saturation isotherms at 25 °C by three separate methods of determination (Figs. 2 and 3). The B_{max} value for [^{3}H]DTZ323 binding measured at 37 °C was similar to the value previously reported for [^{3}H]diltiazem, [^{3}H]isradipine or [^{3}H]D888 (Hagiwara et al., 1997). In the competition experiments, diltiazem competitively inhibited specific [^{3}H]DTZ323 binding. Stereospecificity in [^{3}H]DTZ323 binding was observed with DTZ323 and its stereoisomer (Fig. 4 and Table 1). The potency of DTZ323 compared to diltiazem (=51-fold at 25 °C, =54-fold at 37 °C; Tables 1 and 2) was similar to that (=48-fold) obtained in the competition experiments with [^{3}H]diltiazem at 37 °C (Hagiwara et al., 1997). K_d values previously reported for the specific binding of [^{3}H]diltiazem and [^{3}H]azidobutyryl-diltiazem in skeletal muscle membranes were 37 nM at 30 °C (Glossmann et al., 1983) and 86 nM at 0 °C (Narita et al., 1990), respectively. [^{3}H]DTZ323 (K_d=1.4 nM at 25 °C) appeared to be 26 times more potent than the radiolabeled diltiazem derivatives studied in skeletal L-type Ca^{2+} channels. In cardiac muscle membranes, the K_d value for [^{3}H]clentiazem ([^{3}H]TA-3090), another diltiazem derivative, has been reported as 9 nM at 25 °C (Zobrist and Mecca, 1990). The $IC_{50(H)}$ (2.9 nM) and R_H (84%) for high-affinity binding sites in guinea pig cardiac muscles obtained at 1.5 nM [3H]DTZ 323 showed the high-affinity binding of [^{3}H]DTZ323 and DTZ323 to cardiac L-type Ca^{2+} channels, and the binding affinity appeared to be at least three times higher than that of [^{3}H]clentiazem. These results present direct evidence that [^{3}H]DTZ323 selectively binds to the diltiazem-binding site of L-type Ca^{2+}channels with high affinity and that [^{3}H]DTZ323 has properties that make it suitable for ligand-receptor binding studies.

The B_{max} value for [^{3}H]DTZ323 obtained in saturation experiments at 37 °C was smaller than the value at 25 °C. A decrease in B_{max} values at higher temperature has been reported for [^{3}H]diltiazem binding to guinea pig skeletal muscle membranes (Glossmann et al., 1983). The difference may be due to the conformational change of the binding sites into the low-affinity state that could not be detected by the filtration methods, the partial degradation of the ligand binding site at 37 °C, or the higher dissociation rate of [^{3}H]DTZ323 at higher temperatures, potentially resulting in a submaximal occupation of binding sites.

Tetrandrine is a non-benzothiazepine ligand that discriminates between the binding sites for diltiazem and phenylalkylamines by competitively blocking diltiazem binding but only partially blocking phenylalkylamine binding (King et al., 1988). Tetrandrine completely inhibited [^{3}H]DTZ323 binding. Interestingly, the potency of tetrandrine in inhibiting [^{3}H]DTZ323 binding increased as the temperature was raised from 25 °C (K_i=113 nM, Table 1) to 37 °C (K_i=61 nM, Table 2). The potency of DTZ323, diltiazem and verapamil was decreased at the higher temperature (Tables 1 and 2). The temperature-dependent increase in the binding affinity of tetrandrine and the temperature-dependent decrease in the affinity of diltiazem are consistent with what was previously reported for [^{3}H]diltiazem (King et al., 1988; Garcia et al., 1986). These results also support the idea that [^{3}H]DTZ323 selectively binds to 1,5-benzothiazepine sites within the Ca^{2+} channel.

Verapamil has been reported to inhibit [^{3}H]diltiazem binding via a negative allosteric interaction (Garcia et al., 1986; Balwierczak et al., 1987). Verapamil inhibited [^{3}H]DTZ323 binding in an apparently competitive manner in skeletal and cardiac membranes.

Previously, DTZ323 has been characterized for its affinity and selectivity for 1,5-benzothiazepine site by using [^{3}H]diltiazem, (+)[^{3}H]PN200-110 and (−)[^{3}H]D888 (Hagiwara et al., 1997). The reciprocal allosteric modulation between the diltiazem-binding site and the dihydropyridine-binding site has been reported to vary, among the derivatives, from potentiation to inhibition and is largely dependent on the temperature (Narita et al., 1990; Striessnig et al., 1990; Ikeda et al., 1991). For instance, diltiazem enhances [^{3}H]dihydropyridine binding at 37 °C but inhibits it at 2 °C (Ikeda et al.,

1991). We have previously reported that DTZ323 exerts an inhibitory effect on [^{3}H]dihydropyridine binding at 37 °C (Hagiwara et al., 1997). In the present study, we showed that nicardipine allosterically inhibited [^{3}H]DTZ323 binding at 37 °C in a Ca^{2+}-dependent manner.

It is known that Ca^{2+} decreases the affinity of benzothiazepines and phenylalkylamines for their receptor sites on L-type Ca^{2+} channels, whereas it increases the binding affinity of dihydropyridines for brain and heart membranes, and for solubilized skeletal muscle membranes (Glossmann and Ferry, 1985). In the present study, addition of 1 mM Ca^{2+} to the incubation mixture significantly reduced the affinity of [^{3}H]DTZ323 binding without changing the B_{max} value. Addition of 1 mM Ca^{2+} also induced a rightward shift in the inhibition curves of [^{3}H]DTZ323 binding by DTZ323, diltiazem, tetrandrine or verapamil. The addition of 1 mM Ca^{2+} decreased the IC_{50} value for the inhibition of [^{3}H]DTZ323 binding by nicardipine, presumably due to an increase in the affinity of nicardipine for its own binding site. These differences in the modulation by Ca^{2+} of binding affinity among Ca^{2+} channel antagonists may reflect the difference in their binding sites within the L-type Ca^{2+} channel α_{1C} subunit (Adachi-Akahane and Nagao, 2000), among which the dihydropyridine-binding site appears to be affected by the pore-forming IIIS5-S6 linker region while that is not clearly the case for the diltiazem-binding site (Yamaguchi et al., 2000).

Differences in K_d and B_{max} values were observed between the competition experiments (pseudo-Scatchard analysis) and the saturation experiments at 37 °C. The difference may have originated from the concentration of [^{3}H]DTZ323 (4 nM; 2-fold higher than the K_d value) and the concentration of unlabeled DTZ323 (10 μM; 10-fold higher than that used in other competition experiments) (i.e., Fig. 4) used in the competition experiments to detect the rightward shift of the inhibition curve induced by the addition of 1 mM Ca^{2+}. These conditions, which were specifically designed to detect the Ca^{2+} dependence of [^{3}H]DTZ323 binding, may have seriously affected the analysis of binding parameters. The Ca^{2+} dependence of the allosteric modulation itself, however, was successfully evaluated.

The high-affinity site for Ca^{2+} channel antagonist ligands in cardiac muscle is associated with L-type Ca^{2+} channels (Brush et al., 1987; Zernig and Glossmann, 1988). DTZ323, DTZ417, L-*cis*-DTZ323 and diltiazem inhibited specific [^{3}H]DTZ323 binding to the high-affinity sites in crude guinea pig ventricular membranes, with an IC_{50} value consistent with that obtained for skeletal muscle L-type Ca^{2+} channels. The binding of diltiazem to L-type Ca^{2+} channels is reported to be highly stereospecific in favor of the D-*cis* isomer (Ikeda et al., 1991). A similar stereospecificity in favor of the D-*cis* isomer of DTZ323 was also observed in both skeletal muscle membranes and cardiac membranes containing high-affinity [^{3}H]DTZ323-binding sites.

It is reported that the potency of DTZ323 in inhibiting $I_{Ca(L)}$ in guinea pig ventricular myocytes is 24 times higher than that of diltiazem and that the potency of DTZ417 is comparable to that of diltiazem (Kurokawa et al., 1997b). The potency of DTZ323 compared to that of diltiazem (=22-fold) and the potency of DTZ417 compared to that of diltiazem (=1.1-fold) obtained in this study with high-affinity binding sites in cardiac membranes are consistent with the results obtained in electrophysiological experiments. The results present direct evidence that DTZ323 and DTZ417 inhibit $I_{Ca(L)}$ by their specific binding to L-type Ca^{2+} channels in guinea pig ventricular myocytes.

In crude guinea pig ventricular membranes, the low-affinity site is distinct from the high-affinity site and has been associated with mitochondrial membrane contaminants by using radiolabeled dihydropyridine, such as [^{3}H]nitrendipine (Brush et al., 1987; Zernig and Glossmann, 1988). The low-affinity site observed in this study, therefore, could be due to the presence of contaminants other than sarcolemmal membranes.

In conclusion, our results indicate that [^{3}H]DTZ323 is the most potent radioligand for the diltiazem-binding site in the L-type Ca^{2+} channel among the 1,5-benzothiazepine radioligands studied so far, and that its binding properties make it suitable for ligand-receptor binding studies. The presented results support the electrophysiological data that DTZ323 and DTZ417 inhibit $I_{Ca(L)}$ by their specific binding to the diltiazem-binding sites in guinea pig cardiac L-type Ca^{2+} channels. The high-affinity characteristics of [^{3}H]DTZ323, DTZ323 and DTZ417 are expected to facilitate ligand screening and further characterization of the 1,5-benzothiazepine-binding site on the voltage-dependent L-type Ca^{2+} channel α_{1C} subunit. This new ligand will be of great help in elucidating the molecular mechanisms underlying the tissue selectivity of Ca^{2+} channel antagonists, which is the basis of their clinical usefulness.

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References

Adachi-Akahane, S., Nagao, T., 2000. Ca^{2+} Channel antagonists and agonists. In: Endo, M., Kurachi, Y., Mishina, M. (Eds.), Pharmacology of Ionic Channel Function: Activators and inhibitors. Handbook of Experimental Pharmacology, vol. 147. Springer-Verlag, Berlin, pp. 119–154.

Balwierczak, J.L., Johnson, C.L., Schwartz, A., 1987. The relationship between the binding site of [^{3}H]D-*cis*-diltiazem and that of other non-

dihydropyridine calcium entry blockers in cardiac sarcolemma. Mol. Pharmacol. 31, 175–179.

Brush, K.L., Perez, M., Hawkes, M.J., Pratt, D.R., Hamilton, S.L., 1987. Low affinity binding sites for 1,4-dihydropyridines in mitochondria and in guinea pig ventricular membranes. Biochem. Pharmacol. 36, 4153–4161.

Catterall, W.A., Striessnig, J., 1992. Receptor sites for Ca^{2+} channel antagonists. Trends Pharmacol. Sci. 13, 256–262.

Cheng, Y.C., Prusoff, W.H., 1973. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Ehlert, F.J., 1988. Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. Mol. Pharmacol. 33, 187–194.

Garcia, M.L., King, V.F., Siegl, P.K., Reuben, J.P., Kaczorowski, G.J., 1986. Binding of Ca^{2+} entry blockers to cardiac sarcolemmal membrane vesicles: characterization of diltiazem-binding sites and their interaction with dihydropyridine and aralkylamine receptors. J. Biol. Chem. 261, 8146–8157.

Glossmann, H., Ferry, D.R., 1985. Assay for calcium channels. Methods Enzymol. 109, 513–550.

Glossmann, H., Linn, T., Rombusch, M., Ferry, D.R., 1983. Temperature-dependent regulation of D-*cis*-[^{3}H]diltiazem binding to Ca^{2+} channels by 1,4-dihydropyridine channel agonists and antagonists. FEBS Lett. 160, 226–232.

Hagiwara, M., Adachi-Akahane, S., Nagao, T., 1997. High-affinity binding of DTZ323, a novel derivative of diltiazem, to rabbit skeletal muscle L-type Ca^{++} channels. J. Pharmacol. Exp. Ther. 281, 173–179.

Ikeda, S., Oka, J.-I., Nagao, T., 1991. Effects of four diltiazem stereoisomers on binding of D-*cis*-[^{3}H]diltiazem and (+)-[^{3}H]PN200-110 to rabbit T-tubule calcium channels. Eur. J. Pharmacol. 208, 199–205.

Kanda, S., Adachi-Akahane, S., Nagao, T., 1998. Functional interaction between benzothiazepine- and dihydropyridine binding sites of cardiac L-type Ca^{2+} channels. Eur. J. Pharmacol. 358, 277–287.

King, V.F., Garcia, M.L., Himmel, D., Reuben, J.P., Lam, Y.K., Pan, J.X., Han, G.Q., Kaczorowski, G.J., 1988. Interaction of tetrandrine with slowly inactivating calcium channels. Characterization of calcium channel modulation by an alkaloid of Chinese medicinal herb origin. J. Biol. Chem. 263, 2238–2244.

Kurokawa, J., Adachi-Akahane, S., Nagao, T., 1997a. Effect of a novel, potent benzothiazepine Ca^{2+} channel antagonist, DTZ323, on guinea-pig ventricular myocytes. Eur. J. Pharmacol. 325, 229–236.

Kurokawa, J., Adachi-Akahane, S., Nagao, T., 1997b. 1,5-Benzothiazepine binding domain is located on the extracellular side of the cardiac L-type Ca^{2+} channel. Mol. Pharmacol. 51, 262–268.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mitterdorfer, J., Grabner, M., Kraus, R.L., Hering, S., Prinz, H., Glossmann, H., Striessnig, J., 1998. Molecular basis of drug interaction with L-type Ca^{2+} channels. J. Bioenerg. Biomembranes 30, 319–334.

Naito, K., McKenna, E., Schwartz, A., Vaghy, P.L., 1989. Photoaffinity labeling of the purified skeletal muscle calcium antagonist receptor by a novel benzothiazepine, [^{3}H]azidobutyryl diltiazem. J. Biol. Chem. 264, 21211–21214.

Narita, H., Gaino, M., Suzuki, T., Kurosawa, H., Inoue, H., Nagao, T., 1990. Synthesis and pharmacological properties of azido derivatives of 1,5-benzothiazepine Ca antagonist. Chem. Pharm. Bull. 38, 407–410.

Okuyama, R., Adachi-Akahane, S., Nagao, T., 1994. Differential potentiation by depolarization of the effects of calcium antagonists on contraction and Ca^{2+} current in guinea-pig heart. Br. J. Pharmacol. 113, 451–456.

Spedding, M., Paoletti, R., 1992. Classification of calcium channels and the sites of action of drugs modifying channel function. Pharmacol. Rev. 44, 363–376.

Striessnig, J., Scheffauer, F., Mitterdorfer, J., Schirmer, M., Glossmann, H., 1990. Identification of the benzothiazepine-binding polypeptide of skeletal muscle calcium channels with (+)-*cis*-azidodiltiazem and anti-ligand antibodies. J. Biol. Chem. 265, 363–370.

Tomlinson, G., Hnatowich, M.R., 1988. Apparent competitive inhibition of radioligand binding to receptors: experimental and theoretical consideration in the analysis of equilibrium binding data. J. Recept. Res. 8, 809–830.

Triggle, D.J., 1991. Calcium-channel drugs: structure-function relationships and selectivity of action. J. Cardiovasc. Pharmacol. 18 (Suppl. 10), S1–S6.

Yamaguchi, S., Okamura, Y., Nagao, T., Adachi-Akahane, S., 2000. Serine residue in IIIS5-S6 linker of L-type Ca^{2+} channel α_{1C} subunit is the critical determinant of the action of dihydropyridine Ca^{2+} channel agonists. J. Biol. Chem. 275, 41504–41511.

Zernig, G., Glossmann, H., 1988. A novel 1,4-dihydropyridine-binding site on mitochondrial membranes from guinea-pig heart, liver and kidney. Biochem. J. 253, 49–58.

Zobrist, R.H., Mecca, T.E., 1990. [^{3}H]TA-3090, a selective benzothiazepine-type calcium channel receptor antagonist: in vitro characterization. J. Pharmacol. Exp. Ther. 253, 461–465.