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Diltiazem derivatives modulate the dihydropyridine-binding to intact rat ventricular myocytes

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Abstract

To examine whether the modulation of the 1,4-dihydropyridine-binding by diltiazem derivatives, which has been shown in cardiac and skeletal muscle membranes, takes place in intact cardiac myocytes, effects of diltiazem derivatives on the specific binding of [^{3}H](+)-PN200-110 to freshly isolated adult rat ventricular myocytes were investigated in normal Tyrode solution at 37°C. Diltiazem consistently potentiated the [^{3}H](+)-PN200-110-binding in a concentration-dependent manner, while DTZ323 (3-(acetyloxy)-5-[2-[[2-(3,4-dimethoxyphenyl)ethyl]-methylamino]ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5*H*)-one), a potent diltiazem derivative, inhibited it in a non-competitive manner. In saturation studies, 100 μM diltiazem decreased the K_d value of the [^{3}H](+)-PN200-110-binding (control, 0.102 ± 0.008 vs. diltiazem, 0.074 ± 0.004 (nM, $n = 6$), $P < 0.05$) without significant effect on B_{max} (control, 65.7 ± 6.4 vs. diltiazem, 76.7 ± 4.4 (fmol/mg protein, $n = 6$)). Moreover, membrane-impermeant quaternary diltiazem also potentiated the [^{3}H](+)-PN200-110-binding in intact myocytes. These results suggest that diltiazem modulates the 1,4-dihydropyridine-binding even in intact cardiac myocytes, and that the binding site of diltiazem is accessible from the extracellular side of the L-type Ca^{2+} channels.

Keywords: Diltiazem; 1,4-Dihydropyridine; Ca^{2+} channel, L-type; Ca^{2+} channel antagonist; Cardiac myocyte; Ventricular myocyte; (Rat)

1. Introduction

Ca^{2+} channel antagonists have been studied extensively as useful probes for investigating the functional role of the voltage-dependent L-type Ca^{2+} channels and their molecular structure. Three major groups of Ca^{2+} channel antagonists, i.e., 1,4-dihydropyridines (e.g., nifedipine, nitrendipine), phenylalkylamines (e.g., verapamil), and 1,5-benzothiazepines (e.g., diltiazem), have been proved to act selectively on the voltage-dependent L-type Ca^{2+} channels and to have distinct binding sites on the α_1 subunit of L-type Ca^{2+} channels (Glossmann and Striessnig, 1990; Catterall and Striessnig, 1992). It has also been shown that the separate binding sites for these compounds interact with each other allosterically. A typical example is the potentiation of the 1,4-dihydropyridine-binding by diltiazem at 37°C (DePover et al., 1982; Yamamura et al., 1982; Glossmann et al., 1983, 1985; Vaghy et al., 1987). This potentiation was observed in membrane preparations obtained from various tissues including skeletal muscle, cardiac muscle, brain, and has been widely accepted to be the characteristic feature of 1,5-benzothiazepines. However, certain 1,5-benzothiazepines (e.g., azidobutyryl-diltiazem, DTZ323) have recently been reported to inhibit the 1,4-dihydropyridine-binding (Narita et al., 1990; Nagao et al., 1994).

Most of the binding studies reported so far have been performed in a cell-free system using membrane preparations. However, the affinity of 1,4-dihydropyridines to Ca^{2+} channels has been shown to be higher at depolarized membrane potentials and to vary depending on the membrane potential of myocytes (Bean, 1984; Morel and Godfraind, 1991). Thus, whether the positive allosteric modulation of the 1,4-dihydropyridine-binding to Ca^{2+} channels by diltiazem derivatives takes place in intact myocytes at physiological resting membrane potential was questioned in the present study. Some investigators have attempted to quantify the voltage-dependence of the binding of Ca^{2+} channel antagonists and their allosteric interactions in intact cell preparations (Kokubun et al., 1986; Porzig and

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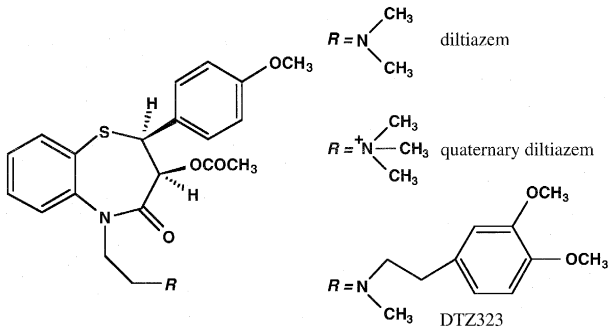

Fig. 1. Chemical structures of 1,5-benzothiazepine derivatives used in the present study.

Becker, 1988; Wei et al., 1989). However, accurate measurement of saturation binding of Ca^{2+} channel antagonists to intact cells had not been performed.

The critical domain within the α_1 subunit of the L-type Ca^{2+} channel for the binding of 1,4-dihydropyridines and phenylalkylamines have been well studied (Catterall and Striessnig, 1992; Varadi et al., 1995). On the other hand, the binding domain for 1,5-benzothiazepines remains to be clarified (Adachi-Akahane et al., 1993; Adachi-Akahane and Nagao, 1993; Naito et al., 1989). If the allosteric effects of 1,5-benzothiazepines on the 1,4-dihydropyridine-binding are observed in intact myocytes, such experiments as to examine the effect of a membrane-impermeant quaternary derivative of diltiazem would give a strong evidence for determining the locus of the 1,5-benzothiazepine-binding site within the α_1 subunit. Therefore, in this study, we investigated the interaction between the binding sites of 1,4-dihydropyridines and 1,5-benzothiazepines using isolated intact adult rat ventricular myocytes which have been well characterized in electrophysiological studies. As specific ligands for the 1,5-benzothiazepine-site, we used *d-cis*-diltiazem, its quaternary derivative, and DTZ323 (3-(acetyloxy)-5-[2-[[2-(3,4-dimethoxyphenyl)ethyl]-methylamino]ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5*H*)-one, see Fig. 1) which we have reported to be 30 times as potent as *d-cis*-diltiazem in both radioligand binding and electrophysiological studies (Nagao et al., 1994).

The aim of this study was (1) to clarify the nature of the interaction between 1,4-dihydropyridine- and 1,5-benzothiazepine-binding sites in intact cardiac myocytes, (2) to determine the sidedness of the 1,5-benzothiazepine-binding site using quaternary diltiazem, permanently charged membrane-impermeant derivative of diltiazem. The preliminary data of the present study has been presented in abstract form (Kanda et al., 1996).

2. Materials and methods

2.1. Animals and chemicals

Male Wistar rats (Crj-Wistar, 250–300 g) were obtained from Charles River Japan Co. (Atsugi, Japan). [^{3}H](+)-PN200-110 (83 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, USA). Diltiazem, quaternary diltiazem and DTZ323 were generous gifts from Tanabe Seiyaku (Saitama, Japan). (±)-Nitrendipine was a gift from Yoshitomi Pharmaceutical (Osaka, Japan). Collagenase (collagenase S-1) was purchased from Nitta Gelatin (Osaka, Japan). Trypsin and Trypsin inhibitor (type III-O) were purchased from Sigma (St. Louis, MO, USA). All other materials were of reagent grade quality and obtained from standard sources. The chemical structures of the diltiazem derivatives are shown in Fig. 1.

2.2. Isolation of ventricular myocytes

Rat ventricular myocytes were prepared following the procedure of Kitagawa et al. (1995) with a slight modification. A rat was heparinised (1000 U/kg) and anaesthetised with sodium pentobarbital (50 mg/kg). The heart was rapidly excised, placed in ice-cold Tyrode solution (NaCl 135 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, Hepes 5 mM, glucose 5.8 mM, pH 7.4) containing 2.6 mM EGTA, and attached to a Langendorff apparatus. All of the solutions used for perfusion were kept at 37°C and gassed continuously with 100% O_2. The perfusion was performed with Tyrode solution containing 2.6 mM EGTA for 2 min, high-K^+ solution (NaCl 4 mM, K-glutamate 130 mM, KCl 4 mM, $CaCl_2$ 20 μM, $MgCl_2$ 1 mM, Hepes 10 mM, glucose 10 mM, pH 7.4) for 5 min, high-K^+ solution containing trypsin (100 U/ml) for 15 min, and then with high-K^+ solution containing collagenase (350 U/ml) for 14–17 min, followed by high-K^+ solution for 2 min to wash out the enzyme. The ventricles were cut into 5–6 sections, and placed in 30 ml high-K^+ solution containing trypsin inhibitor (0.24 mg/ml) and bovine serum albumin (1 mg/ml). The ventricle sections were incubated at 37°C for 10 min with gentle shaking (120 cycles/min). The resulting cell suspension was filtered, then incubated for 20 min at room temperature and centrifuged at $50 \times g$ for 60 s. The pellet was resuspended in Hepes-buffered Joklik-modified Minimum Essential Medium (Hepes-MEM) containing 2 mM $CaCl_2$ and 10 mM Hepes ($NaHCO_3$ was substituted with equimolar NaCl), and incubated at room temperature for 20 min to select Ca^{2+}-tolerant cells. The cell suspension was then layered on a Percoll gradient preformed by centrifugation of 50% (v/v) Hepes-MEM-Percoll solution (pH 7.2) at $30\,000 \times g$ for 7 min. This Percoll gradient was then centrifuged at $1500 \times g$ for 5 min. Isolated myocytes were collected from the band at a density of 1.063–1.075 (g/ml), and centrifuged at $50 \times g$ for 2 min. Finally, we obtained $(1–2) \times 10^6$ myocytes, more than 80% of which excluded trypan blue. However, we often found shrunk or round-edged myocytes, which are signs of Ca^{2+}-overload of myocytes, even in Trypan blue-resistant cells. Thus, we checked the viability of the isolated myocytes with stricter criteria such as rod-shape with sharp edge and clear striation. We

checked the time-dependent change of viability of myocytes, and confirmed that the loss of viability of myocytes during two hours of experimental protocol was less than 10%. The following experiments were carried out only when the yield of rod-shaped and clearly striated myocytes exceeded 75%.

2.3. Binding assays

Radioligand binding studies were carried out using [^{3}H](+)-PN200-110. For both saturation and competition studies, cell suspension (approximately 20 000 myocytes/tube) was incubated with the radioligand and drugs in a total volume of 300 μl. Protein concentration determined by the Lowry method was 0.75–1.75 mg/ml. The concentration of [^{3}H](+)-PN200-110 was 0.05–1 nM in saturation studies and 0.15 nM in competition studies. Incubation was carried out for 60 min at 37°C. The binding reaction was terminated by the addition of the ice-cold Tyrode solution, followed by rapid vacuum filtration using a cell harvester (model M-24, Brandel) through Whatman GF/C glass fibre filters which were presoaked with 0.5% polyethyleneimine and 0.2% bovine serum albumin for at least 2 h before use. The filters were washed with an additional 5 ml of Tyrode solution for three times, and then incubated in 4 ml of scintillation cocktail (Clear-sol II, Nakalai Tesque) overnight. The radioactivity was determined with liquid scintillation counter.

All determinations were performed in duplicate. Non-specific binding was determined with 1 μM (±)-nitrendipine. All drugs were diluted with Tyrode solution. (±)-Nitrendipine at 3×10^{-3} M was dissolved in absolute ethanol to prepare stock solution, and was diluted with Tyrode solution so that the final ethanol concentration in the incubation solution was less than 0.1%. DTZ323 stock solution at a concentration of 10^{-3} M was prepared by dissolving it in distilled water. Binding assays were performed in the dark to avoid the breakdown of light-sensitive 1,4-dihydropyridine ligands.

2.4. Data analysis

All binding parameters were calculated by computer fitting (GraphPad, InPlot).

K_d and B_{max} values were calculated by fitting the data obtained in the equilibrium saturation studies to the Michaelis-Menten equation by non-linear regression analysis with weight values of $1/y^2$.

IC_{50} values, slope factors, and the maximum effects of (±)-nitrendipine and DTZ323 were estimated by fitting the experimental data obtained in competition studies to the logistic equation by non-linear regression analysis. A value of inhibition constant (K_i) for (±)-nitrendipine was calculated from IC_{50} value using the following equation:

$K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of radioligand and K_d is the equilibrium dissociation constant of [^{3}H](+)-PN200-110 obtained from saturation studies.

Results were expressed as means ± S.E.M. of 5–6 experiments. Statistical significance was assessed with Student-Welch's *t*-test. Differences at $P < 0.05$ were considered to be significant.

3. Results

3.1. Specific binding of [^{3}H](+)-PN200-110 to adult rat ventricular myocytes

Fig. 2 shows a typical example of the equilibrium saturation binding of [^{3}H](+)-PN200-110 to isolated adult rat ventricular myocytes. The specific binding of [^{3}H](+)-PN200-110 was saturable in the concentration range of 0.05–1 nM, and the non-specific binding was linearly dependent on the concentration of the labelled ligand. However, when the concentration of the labelled ligand exceeded 1 nM, the specific binding started to slightly increase again, as if there were low-affinity site-like component. It was more obvious in a saturation study in the presence of 100 μM diltiazem. This component might be attributed to the large non-specific binding with the labelled ligand exceeding 1 nM. Thus, we omitted this component in the following analysis. The average values for K_d and B_{max} of the [^{3}H](+)-PN200-110-binding derived from the equilibrium saturation studies are summarized in Table 2.

3.2. Effects of diltiazem and DTZ323 to the specific binding of [^{3}H](+)-PN200-110

In competition studies, [^{3}H](+)-PN200-110 was used at the concentration close to the K_d value derived from the

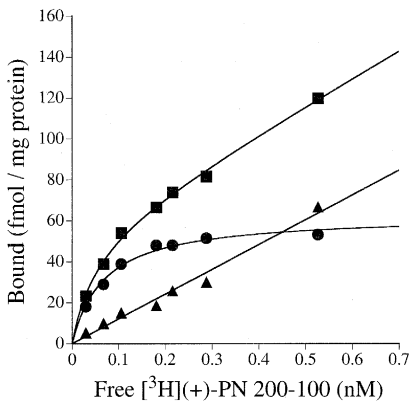

Fig. 2. Equilibrium saturation binding of [^{3}H](+)-PN200-110 to isolated rat ventricular myocytes. Total (■), specific (●) and non-specific (▲) binding are shown. Non-specific binding was determined in the presence of 1 μM (±)-nitrendipine. Points are means of duplicate determinations from one representative experiment. The K_d and B_{max} values obtained from this fitting curve were 0.074 nM and 63.8 fmol/mg protein, respectively.

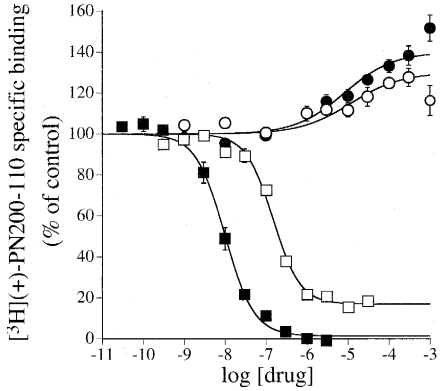

Fig. 3. Competition of diltiazem (●), quaternary diltiazem (○), DTZ323 (□) and (±)-nitrendipine (■) with the specific binding of [^{3}H](+)-PN200-110 to isolated rat ventricular myocytes. Each data points are normalized relative to the [^{3}H](+)-PN200-110-binding to myocytes measured in the absence of drugs as 100%. Each plot represents mean ± S.E.M. of 5–6 experiments. Fitting lines were created so as to fit by eye.

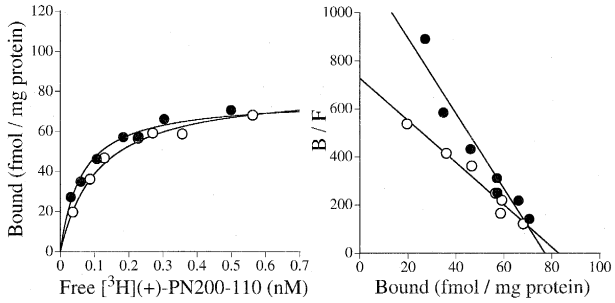

Fig. 4. Effect of diltiazem on the equilibrium saturation binding of [^{3}H](+)-PN200-110 to isolated ventricular myocytes. Left panel shows the typical results of the equilibrium saturation binding of [^{3}H](+)-PN200-110. Equilibrium binding studies with [^{3}H](+)-PN200-110 were carried out in the absence (○) and presence of 100 μM diltiazem (●). Non-specific binding was determined in the presence of 1 μM (±)-nitrendipine. Points of the respective groups are means of duplicate determination of single representative experiment. Right panel shows the Scatchard plot of the data shown in the left panel. The K_d (nM) and B_{max} (fmol/mg protein) values were estimated to be 0.111 and 81.8 in the absence of diltiazem (control), 0.063 and 76.2 in the presence of diltiazem, respectively.

saturation studies, 0.15 nM. Each point of the competition curve was normalized relative to the specific binding of [^{3}H](+)-PN200-110 measured in the absence of drugs as 100%. The specific binding represented as 100% was 27.4 ± 1.1 fmol/mg protein ($n = 11$).

Fig. 3 and Table 1 show the results of the competition studies. (±)-Nitrendipine inhibited the specific binding of [^{3}H](+)-PN200-110 completely with a slope factor of approximately 1 and the K_i value of 4.8 nM. Diltiazem potentiated the specific binding of [^{3}H](+)-PN200-110 in a concentration-dependent manner up to 135%. The results with 10^{-3} M diltiazem varied: the binding increased in 4 among 5 experiments and decreased in one experiment. In contrast to diltiazem, DTZ323 suppressed the specific binding of [^{3}H](+)-PN200-110 to 17% in a non-competitive manner with a slope factor of 1.5.

3.3. Effect of diltiazem on the equilibrium saturation binding of [^{3}H](+)-PN200-110

Equilibrium saturation binding studies were performed in the presence or absence of diltiazem (Fig. 4, Table 2). Myocytes were incubated with 100 μM diltiazem which potentiated the binding of [^{3}H](+)-PN200-110 up to 130% in competition studies (Fig. 3). Diltiazem significantly decreased the equilibrium dissociation constant, K_d, but did not give significant effect on the maximum binding density, B_{max}, of the [^{3}H](+)-PN200-110-binding. Diltiazem did not change the shape of the saturation curve. In fact, when we calculated B_{max} value with respect to the number of cells, B_{max} values in the absence or presence of diltiazem were 6.06 ± 0.10 and 8.97 ± 0.53 fmol/10^4 myocytes, respectively. In this case, B_{max} value calculated in the presence of diltiazem was significantly larger than that in the absence of diltiazem ($P < 0.01$). But, considering the accuracy of cell counting, we relied on B_{max} values expressed in terms of protein contents of myocytes (fmol/mg protein).

3.4. Effect of quaternary diltiazem on the specific binding of [^{3}H](+)-PN200-110 to intact myocytes

As is shown in Fig. 3 and Table 1, quaternary diltiazem potentiated the specific binding of [^{3}H](+)-PN200-110 in

Table 1
Effects of 1,5-benzothiazepine derivatives and (±)-nitrendipine on the specific binding of [^{3}H](+)-PN200-110 to isolated rat ventricular myocytes

	IC_{50} (nM)	Slope factor	Maximum effect (%)	*n*
Diltiazem	–	–	138 ± 5	5
Quaternary diltiazem	–	–	128 ± 4	6
DTZ323	165 ± 13	1.57 ± 0.11	17.2 ± 1.0	5
Nitrendipine	9.52 ± 1.77	1.15 ± 0.08	–	5

Each value represents mean ± S.E.M.

Table 2
Effect of diltiazem on the equilibrium saturation binding parameters of [^{3}H](+)-PN200-110 binding to isolated rat ventricular myocytes

	K_d (nM)	B_{max} (fmol/mg protein)	*n*
Control	0.102 ± 0.008	65.7 ± 6.4	6
Diltiazem	0.074 ± 0.004 [a]	76.7 ± 4.4	6

Equilibrium saturation binding studies with [^{3}H](+)-PN200-110 were carried out in the absence (control) and presence of 100 μM diltiazem. Non-specific binding was determined in the presence of 1 μM (±)-nitrendipine. Each value represents the mean ± S.E.M. [a] $P < 0.05$ vs. control.

a concentration-dependent manner, although at approximately 10 times higher concentration compared to diltiazem. Results with 10^{-3} M quaternary diltiazem also varied as was the case with 10^{-3} M diltiazem.

4. Discussion

In the present study, we examined the specific binding of 1,4-dihydropyridine Ca^{2+} channel antagonist and interaction between 1,4-dihydropyridine- and 1,5-benzothiazepine-sites using intact rat ventricular myocytes bathed in physiological Tyrode solution containing 2 mM Ca^{2+}, 1 mM Mg^{2+} and 5.4 mM K^+. The specific binding of [^{3}H](+)-PN200-110 to isolated adult rat ventricular myocytes was saturable with the concentration ranging between 0.05–1 nM. Scatchard analysis of [^{3}H](+)-PN200-110-binding indicated a single population of non-cooperative binding sites. These results are in contrast to the saturation binding studies in which the specific binding of [^{3}H](+)-PN200-110 to primary-cultured neonatal rat ventricular myocytes did not saturate under the condition similar to ours (Porzig and Becker, 1988; Wei et al., 1989). The discrepancy of the results may be due to the purity of viable ventricular myocytes. We could obtain ventricular myocytes of nearly identical quality with high viability, which probably enabled us to observe sufficiently saturable binding in this study. Accordingly, we confirmed that the binding assay system used in this study is useful for quantifying the binding under physiological condition. In this study, the obtained K_d value for [^{3}H](+)-PN200-110, 0.102 nM, was about 1.5 times larger than that reported by Lee et al. (0.064 nM) in rat cardiac membranes (Lee et al., 1984). The affinity of [^{3}H](+)-PN200-110 for intact ventricular myocytes may be low at the resting membrane potential.

In competition studies, K_i value for (±)-nitrendipine was approximately 5 nM. Compared with K_d value for [^{3}H](+)-PN200-110 (about 0.1 nM), this indicates that the affinity for (±)-nitrendipine was 50 times as low as that for (+)-PN200-110, which is similar to the difference of K_d values reported in rat cardiac membranes (Lee et al., 1984). Diltiazem at 10^{-10}–10^{-4} M has been reported to potentiate the binding of [^{3}H](+)-PN200-110 up to 145% in rat cardiac membranes (Lee et al., 1984). This is in good agreement with our data showing that diltiazem potentiated the binding of [^{3}H](+)-PN200-110 up to about 140%. Therefore, it is demonstrated that the potentiation of the binding of 1,4-dihydropyridine induced by diltiazem observed in membrane preparations is also observed in intact ventricular myocytes under physiological conditions. In contrast to diltiazem, DTZ323 partially inhibited the binding of [^{3}H](+)-PN200-110 (Fig. 3, Table 1). This result is also consistent with our previous study in skeletal muscle membranes (Nagao et al., 1994). Effects of DTZ323 on association and dissociation rate constants need to be examined for clarifying this issue. In fact, it has been shown that *d-cis*-diltiazem decreases both association and dissociation rate constants, and that *l-cis*-diltiazem, which partially inhibit the binding of 1,4-dihydropyridines, increases the dissociation rate constant but has no effect on the association rate constant (Ikeda et al., 1991). Therefore, the balance of the effects on the association and dissociation rate constants appears to determine whether to increase or decrease the 1,4-dihydropyridine-binding. These results suggest that the ability to potentiate the binding of 1,4-dihydropyridines is not an intrinsic property of all of 1,5-benzothiazepines.

In saturation studies, 100 μM diltiazem decreased the K_d value of [^{3}H](+)-PN200-110 by 30% and slightly increased B_{max}, which is consistent with pervious reports (Lee et al., 1984; Ikeda et al., 1991). In the study using primary-cultured rat neonatal ventricular myocytes, it was reported that diltiazem decreased the K_d value of [^{3}H](+)-PN200-110 without affecting the B_{max} value under the condition similar to this study (Porzig and Becker, 1988). However, in their study, the B_{max} and K_d values were estimated by extrapolation of the very initial part of the saturation curve. Thus more accurate calculation of the K_d and B_{max} values needed to be carried out. In this study, we accurately showed effects of diltiazem on the K_d and B_{max} values for the [^{3}H](+)-PN200-110-binding. Consequently, our results indicate that the binding affinity of 1,4-dihydropyridines to the functional Ca^{2+} channels is also potentiated by diltiazem under physiological resting condition. On the basis of binding studies, this potentiation by diltiazem has been postulated to be caused by the shift of the channel gating by diltiazem to the inactivated state to which 1,4-dihydropyridine has high affinity (Glossmann et al., 1985). Porzig and Becker found that this potentiation was abolished by depolarization induced by high K^+ in primary-cultured neonatal rat ventricular myocytes (Porzig and Becker, 1988). Accordingly, they concluded that the inactivated state of channels induced by diltiazem was different from that induced by depolarization. At a single channel level, diltiazem has been electrophysiologically shown to interact with the inactivated state of the channel (Zahradníková and Zahradník, 1992). Whether the inactivation induced by voltage and that induced by diltiazem are different is to be examined in the future.

We calculated the B_{max} value using the counted number of myocytes, and then calculated Ca^{2+} channel density to be 3.6 and 5.4×10^5 channels/myocyte in the absence and the presence of 100 μM diltiazem, respectively. We also calculated the channel density based on the idea that whole-cell charge movement represents the summation of gating charge of individual channels on the plasma membrane, and that six elementary charges move across the membrane in each Ca^{2+} channel (Hill, 1992). Using the saturating gating charge of 550 fC/cell reported in rat ventricular myocytes (Bean and Rios, 1989), the density of

Ca^{2+} channels was calculated to be 5.7×10^5 channels/myocyte. This value corresponds very well with our results. Whether all of these 1,4-dihydropyridine-binding sites represent functional Ca^{2+} channels is unknown. However, it has been shown that the majority of 1,4-dihydropyridine receptors in cardiac myocytes are functional Ca^{2+} channels in rabbit ventricular myocytes (Lew et al., 1991).

The 1,5-benzothiazepine-binding site has been speculated to be accessible from the extracellular side of the membrane in whole cell patch-clamp experiments using benzazepine analogs which potentiate the 1,4-dihydropyridine-binding (Hering et al., 1993). However, the benzazepine analogs are structurally similar but different from 1,5-benzothiazepines. Thus, the 1,5-benzothiazepine-binding site has to be determined with diltiazem analogs.

Quaternary diltiazem has a permanent charge and thus thought to be membrane-impermeant. We reported that quaternary diltiazem inhibit L-type Ca^{2+} channel current in cardiac myocytes and potentiates the binding of 1,4-dihydropyridines like diltiazem in skeletal muscle membrane preparation (Adachi-Akahane et al., 1993). When the sidedness of effect of quaternary diltiazem on L-type Ca^{2+} channel currents was examined by the extracellular and the intracellular application, quaternary diltiazem acted not only from the outside but also from the inside of the myocyte. In this study, quaternary diltiazem given extracellularly in the incubation solution potentiated the binding of [^{3}H](+)-PN200-110 in a manner similar to that of diltiazem. Therefore, given that the potentiation of the 1,4-dihydropyridine-binding by diltiazem is caused via the binding of diltiazem to its specific binding site, the results with quaternary diltiazem give an important implication that the 1,5-benzothiazepine-binding site is accessible from the extracellular side of the membrane. These results are consistent with our finding that diltiazem is 1000 times more potent with the extracellular application than the intracellular application in guinea pig ventricular myocytes (Adachi-Akahane and Nagao, 1993).

Together with these results, we suggest that diltiazem potentiates the specific binding of 1,4-dihydropyridines by binding to the 1,5-benzothiazepine receptor site near the outer side of the membrane under physiological conditions. The electrophysiological relevance of the allosteric interaction between 1,4-dihydropyridine- and 1,5-benzothiazepine-sites needs to be clarified in future studies.

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