Effect of S-312, a New Calcium Channel Blocker, on the 1,4-Dihydropyridine Binding Sites in Porcine Basilar Blood Vessels and Rat Aortic Smooth Muscle Cells

Shin-ichi Mihara and Masafumi Fujimoto*

Shionogi Research Laboratories, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553, Japan

Received October 24, 1990 Accepted March 22, 1991

ABSTRACT—We examined the interaction of two isomers of S-312, a new calcium channel blocker with a bicyclic dihydrothienopyridine structure, with 1,4-dihydropyridine binding sites. Specific bindings of [3H]nitrendipine and (+)[3H]PN200-110 in membranes prepared from porcine basilar blood vessels were saturable, reversible, and stereoselective, and had high affinities. The binding properties were very similar to those in membranes from other tissues such as the aorta, myocardium, and cerebral cortex. 1,4-Dihydropyridine calcium channel blockers competed for each radioligand binding in the order of: nisoldipine = nicardipine = S-(+)-S-312 > nifedipine > R-(−)-S-312. S-(+)-S-312 caused a decrease in the Kd values for both radioligands without changing the maximal binding capacity. 1,4-Dihydropyridines inhibited the high K+-induced increase in cytosolic free Ca2+ concentration in rat aortic smooth muscle A7r5 cells. S-(+)-S-312 was 3.3–4.9 times more potent than nicardipine or nisoldipine in inhibiting the Ca2+ increase, although S-(+)-S-312 bound to A7r5 cells with almost the same affinity. These and earlier findings show that S-(+)-S-312 exerts effects more potent than expected from the affinity for [3H]nitrendipine or (+)-[3H]PN200-110 binding sites. This was the case with R-(−)-S-312. These dihydrothienopyridine derivatives appear to interact with the Ca2+ channel in a manner slightly different from the conventional 1,4-dihydropyridines.

S-312, methyl 4,7-dihydro-3-isobutyl-6-methyl-4-(3-nitrophenyl)thieno[2,3-b]pyridine-5-carboxylate (Fig. 1), was synthesized as one of a new type of 1,4-dihydropyridines with a fused thiophene nucleus (1). This compound has a chiral center and two optical isomers, S-(+)-S-312 and R-(−)-S-312. S-312 shows a calcium antagonist-like pharmacological profile: it inhibits specific [3H]nitrendipine binding in rat cerebral cortex membranes (1); In vivo, it has potent coronary vasodilator and antihypertensive activities (1); and it potently relaxes the helical strips of various isolated rabbit arteries precontracted with high K+-depolarization, serotonin and U46619 (2). Interestingly, S-312 showed a higher activity in basilar arteries than in femoral arteries (2).

1,4-Dihydropyridine Ca2+ channel blocker binding sites were previously identified and characterized in several blood vessels such as the aorta, tail artery and coronary artery (3–7). However, little work has been done on brain blood vessels. Morel and Godfraind have recently revealed the existence of a single class of specific, stereoselective and voltage-dependent binding sites which bind
(+)-[3H]PN200-110 in rat brain microvessels (8). In the present study, we demonstrated the specific binding sites for two 1,4-dihydropyridines, [3H]nitrendipine and (+)-[3H]PN200-110, in membranes from porcine basilar blood vessels and the effect of S-312 and its optical isomers on each binding. We also demonstrated the effect of the two optical isomers on the depolarization-induced increase in cytosolic free Ca^{2+} concentration ([Ca^{2+}]_{j}) in rat aortic smooth muscle A7r5 cells to examine their functional potencies.

MATERIALS AND METHODS

Cell culture and materials

A7r5 cells were obtained from the American Type Culture Collection through Dainippon Seiyaku (Osaka, Japan) and cultured in Medium 199 (GIBCO) supplemented with 10% fetal calf serum (GIBCO), 50 μg/ml streptomycin, and 50 U/ml penicillin G (GIBCO) at 37°C in a fully humidified atmosphere of 5% CO_{2} in air.

S-312 and its optical isomers, nifedipine, nisoldipine, and nicardipine were supplied by Dr. Ikuo Adachi of our laboratories. [3H]-Nitrendipine (3.11 TBq/mmol) and (+)-[3H]-PN200-110 (6.29 TBq/mmol) were purchased from New England Nuclear.

Preparations of membranes

Fresh pig thoracic aortas, brains and hearts were obtained from a local slaughterhouse. Aortic smooth muscle membranes were prepared as described previously (9). Cardiac membranes and membranes from basilar blood vessels were prepared as follows. Porcine cardiac muscles obtained from right and left ventricles were minced with scissors and homogenized with a Polytron homogenizer (Brinkmann Instruments, Inc., Westberg, NY) in sufficient 50 mM Tris-HCl (pH 7.4 at 25°C) so that the final concentration was 200 mg original wet tissue weight/ml buffer. Subsequently, the cardiac homogenates were filtered through four layers of cheesecloth. The filtrates were centrifuged at 77 X g for 10 min, and then the pellet was discarded. The supernatant was centrifuged at 48,000 X g for 15 min. The pellet was washed once and resuspended in Tris buffer (50 mM, pH 7.4), and stored at −80°C until use. Basilar blood vessels were carefully excised with forceps free of any visible non-vascular contaminants. The vessels were minced with scissors and homogenized with the Polytron homogenizer in sufficient 0.25 M sucrose containing 5 mM Tris-HCl (pH 7.4 at 25°C) so that the final concentration was 100 mg original wet tissue weight/ml buffer. The homogenates were filtered through four layers of cheesecloth. The filtrates were centrifuged at 1,000 X g for 10 min. The supernatant was spun at 10,000 X g for 10 min. The final supernatant was then centrifuged at 100,000 X g for 20 min. The pellet was washed once and resuspended in Tris buffer (50 mM, pH 7.4) and stored at −80°C until use. The frozen preparation was thawed, diluted 10- to 15-fold, and used for the binding experiments. Cerebral cortical membranes were prepared by applying the same procedures described above for membranes from basilar blood vessels.

[3H]-Nitrendipine and (+)-[3H]PN200-110 bindings

Ligand binding experiments with mem-
branes were performed according to the method described by Gould et al. (10). The concentrations of membrane protein were within the range in which there was a linear relationship between protein concentration and binding: 150–170, 300–350, 180–210, and 100–200 μg protein/assay for aortic, cardiac, cerebral cortical, and basilar blood vessel membranes, respectively. The incubation mixture (0.5 ml) contained 50 mM Tris-HCl (pH 7.4 at 25°C), various concentrations of [3H]nitrendipine or (+)-[3H]PN200-110, and the displacing compound or vehicle as was appropriate. Incubation was done at 25°C for 50 min for [3H]nitrendipine binding and for 50 or 100 min for (+)-[3H]PN200-110 binding. For the binding experiments with A7r5 cells, cells were dispersed with 0.025% trypsin/1 mM EDTA. Cell suspensions were washed once with the growth medium. Single cells were counted and resuspended in Heps (20 mM)-buffered Hanks’ solution to a final concentration of 1 × 10^6 cells/ml. The cell suspensions were incubated with 2 μM fura-2-AM (Dojin, Kumamoto, Japan) at 37°C for 30 min. The fura-2-loaded cells thus obtained were resuspended in 0.25 ml of Heps (20 mM)-buffered Hanks’ solution (pH 7.4) at 1 × 10^6 cells/ml in a cuvette (7 φ × 50 mm) and continuously stirred. To measure the effect of 70 mM K⁺ on [Ca²⁺], 0.25 ml of 135 mM K⁺ solution (5 mM NaCl, 135 mM KCl, 1.3 mM CaCl₂, 1 mM MgCl₂, 1 mM K₂HPO₄, 10 mM glucose, 20 mM Heps, pH 7.4) was added to the cuvette. Drugs were given in 1 μl dimethyl sulfoxide 30 sec before the addition of the high K⁺ solution. Fluorescence was measured by a spectrofluorometer (CAF-100, Japan Spectroscopy Inc., Tokyo, Japan) as described previously (11).

RESULTS

**Binding of [3H]nitrendipine to membranes from basilar blood vessels**

The binding of [3H]nitrendipine to the membranes from porcine basilar blood vessels was linear over a protein concentration range of 50–200 μg/tube (data not shown). The time course study in the presence of 0.2 nM [3H]nitrendipine showed that binding reached a plateau within 20 min and remained stable for at least 90 min (Fig. 2a). The observed rate constant (k_{obs}), determined from the slope of the pseudo-first order plot (Fig. 2b), was 0.34 min⁻¹. The dissociation rate constant was determined from the time course of the displacement of [3H]nitrendipine from its binding site induced by 10⁻⁶ M nifedipine. A rapid exponential decrease in the binding of [3H]nitrendipine was observed with time (Fig. 2c); the T_{1/2} for the dissociation of the ligand binding complex at 25°C was 6.8 min. Linear transformation of the data yielded a k_{-1} of 0.10 min⁻¹. The dissociation rate constant was determined from the time course of the displacement of [3H]nitrendipine from its binding site induced by 10⁻⁶ M nifedipine. A rapid exponential decrease in the binding of [3H]nitrendipine was observed with time (Fig. 2c); the T_{1/2} for the dissociation of the ligand binding complex at 25°C was 6.8 min. Linear transformation of the data yielded a k_{-1} of 0.10 min⁻¹. The association rate constant (k_1) determined from the equation k_1 = (k_{obs} - k_{-1}) / [L] was found to be 0.53 nM⁻¹ min⁻¹. The kinetic dissociation constant (K_d) given by K_d = k_{-1} / k_1 was found to be 0.19 nM.
Equilibrium binding studies were performed by incubating the membranes with increasing concentrations of \(^{3}H\)nitrendipine (Fig. 3a). As shown in Fig. 3b, Scatchard analysis of the saturation curve showed that the membranes contain a single class of specific binding sites for nitrendipine with an apparent $K_d$ of 0.32 ± 0.02 nM and binding sites ($B_{max}$) of 64 ± 4 fmol/mg protein ($n = 3$). The binding of 0.2 nM \(^{3}H\)nitrendipine was completely and competitively inhibited by 1,4-dihydropyridines, such as nifedipine, nisoldipine and nicardipine (Fig. 4a). The phenylalkylamine verapamil only incompletely inhibited the binding, while the benzothiazepine diltiazem enhanced it at $10^{-7}-10^{-5}$ M (Fig. 4a), as shown previously with the brain, heart, ileum and coronary artery (7, 12–14).

**Effect of two isomers of S-312 and other 1,4-dihydropyridines on \(^{3}H\)nitrendipine binding**

First, we examined the ability of two optical isomers of S-312 to inhibit \(^{3}H\)nitrendipine binding to membranes from basilar blood vessels. S-(+)-S-312 and R-(-)-S-312, like nitrophenyl-1,4-dihydropyridines, completely inhibited the binding, but the affinity of R-(-)-S-312 was 26 times lower than that of S-(+)-S-312 (Fig. 4a and Table 1). As shown in Table 1, S-(+)-S-312 was about 4 times as strong as nifedipine in inhibiting \(^{3}H\)nitrendipine binding, but was a little weaker than nisoldipine or nicardipine. Furthermore, we tested the effect of 1,4-dihydropyridines including two isomers of S-312 on \(^{3}H\)nitrendipine binding in the aorta, myocardium and cerebral cortex in order to examine their tis-
Fig. 3. Saturation bindings of $[3^H]$nitrendipine (NTD) (top) and (+)$[3^H]$PN200-110 (PN) (bottom) to membranes from porcine basilar blood vessels. (a, c): Relationship between $[^{3}H]$ligand concentration and the binding. Membranes (160 or 100 μg protein) were incubated at 25°C for 50 min with $[^{3}H]$nitrendipine in concentrations up to 1 nM or (+)$[^{3}H]$PN200-110 in concentrations up to 400 pM, respectively. Specific binding was obtained by subtracting nonspecific binding from total binding. (b, d): Scatchard plot.

Fig. 4. Effect of various Ca$^{2+}$ channel blockers on $[^{3}H]$nitrendipine (NTD) (a) and (+)$[^{3}H]$PN200-110 (PN) (b) bindings to membranes from porcine basilar blood vessels. Membranes (160 μg protein) were incubated at 25°C with 0.2 nM $[^{3}H]$nitrendipine or 50 pM (+)$[^{3}H]$PN200-110 in the presence of S-(-)-S-312 (●), R-(−)-S-312 (▲), nifedipine (□), nisoldipine (■), nicardipine (○), verapamil (△) and diltiazem (▼) for 50 min. Inhibition of binding was assessed relative to ligand association in untreated membranes. Each $K_i$ value is described in Table 1.
sue selectivity. Table 1 shows that there was practically no difference in $K_i$ values for all 1,4-dihydropyridines between these four membranes.

### Binding of (+)-$[^3]H$PN200-110 to membranes from porcine basilar blood vessels

The membranes from porcine basilar blood vessels bound the other radiolabeled 1,4-dihydropyridine (+)-$[^3]H$PN200-110. Figure 2d shows the association and dissociation of $[^3]H$PN200-110 binding to the membranes. The $k_{obs}$ and $k_{-1}$ were found to be 0.049 min$^{-1}$ and 0.0072 min$^{-1}$, respectively (Fig. 2, c and f). The $k_1$ of 0.41 nM$^{-1}$ min$^{-1}$ was calculated from the above-described equation. The dissociation constant determined kinetically was 18 pM.

The experiment testing the effect of Ca$^{2+}$ channel blockers on (+)-$[^3]H$PN200-110 binding was performed at 25°C for 50 min to compare it with the data obtained with $[^3]H$nitrendipine. Under these conditions, the determined $K_d$ value was 55 ± 11 pM (Fig. 3, c and d). The number of binding sites was 46 ± 10 fmol/mg protein, which was not significantly different from the number of $[^3]H$nitrendipine binding sites. Like $[^3]H$nitrendipine binding, (+)-$[^3]H$PN200-110 binding was completely displaced by 1,4-dihydropyridines and partially displaced by verapamil (Fig. 4b). However, diltiazem inhibited (+)-$[^3]H$PN200-110 binding in contrast to its enhancing effect on $[^3]H$nitrendipine binding (Fig. 4).

### Effect of two isomers of S-312 and other Ca$^{2+}$ channel blockers on (+)-$[^3]H$PN200-110 binding

Figure 4b shows the effect of two optical isomers of S-312 as well as Ca$^{2+}$ channel blockers on (+)-$[^3]H$PN200-110 binding to the membranes, and the $K_i$ values of all 1,4-dihydropyridines tested for inhibition of (+)-$[^3]H$PN200-110 binding were similar to those for inhibition of $[^3]H$nitrendipine binding.

### Table 1. $K_i$ values (nM) for inhibition of $[^3]H$nitrendipine binding in aorta, myocardium, cerebral cortex and basilar blood vessels and of (+)-$[^3]H$PN200-110 ($[^3]H$PN) in basilar blood vessels by calcium channel blockers

| Compound    | Aorta         | Myocardium   | Cerebral cortex | Basilar blood vessels |
|-------------|---------------|--------------|-----------------|-----------------------|
| Nifedipine  | 1.05 ± 0.06   | 0.82 ± 0.10  | 1.08 ± 0.02     | 0.95 ± 0.10           | 1.1 ± 0.0         |
| Nisoldipine | 0.12 ± 0.01   | 0.11 ± 0.02  | 0.12 ± 0.02     | 0.09 ± 0.02           | 0.14 ± 0.01      |
| Nicardipine | 0.20 ± 0.02   | 0.20 ± 0.02  | 0.22 ± 0.05     | 0.21 ± 0.03           | 0.17 ± 0.03      |
| S-312       | 0.40 ± 0.08   | 0.56 ± 0.07  | 0.33 ± 0.06     | 0.31 ± 0.05           | N.D.             |
| S-(+)-S-312 | 0.27 ± 0.05   | 0.29 ± 0.01  | 0.19 ± 0.03     | 0.25 ± 0.05           | 0.14 ± 0.03     |
| R-(−)-S-312 | 8.3 ± 0.4     | 8.4 ± 0.2    | 7.7 ± 0.9       | 6.6 ± 1.4             | 7.5 ± 0.9        |

Each membrane fraction was incubated with 0.2 nM $[^3]H$nitrendipine or 50 pM (+)-$[^3]H$PN200-110 for 50 min at 25°C in the absence or presence of Ca$^{2+}$ channel blockers. $K_i$ values were calculated from the equation $K_i = IC_{50} / (1 + [L]/K_d)$, where $IC_{50} = \text{the concentration causing 50% inhibition of specific }[^3]H\text{ligand binding}$, $[L] = \text{[^3]Hligand concentration}$ and $K_d = \text{dissociation constant for }[^3]H\text{nitrendipine (0.21, 0.27, 0.22 and 0.32 nM in the aorta, myocardium, cerebral cortex and basilar blood vessels, respectively)}$ or for (+)-$[^3]H$PN200-110 (55 pM). IC$_{50}$ values and Hill coefficients were calculated by linear regression from Hill plots of the specific binding data. Hill coefficients were between 0.9 and 1.1. Data represent means ± S.E. (n = 3–4). N.D.: Not determined.
Effect of S-(+)-S-312 on Scatchard plots

To characterize the interaction of S-(+)-S-312 with \(^{3}H\)nitrendipine or (+)-\(^{3}H\)PN200-110 binding sites, saturation binding experiments with \(^{3}H\)nitrendipine were carried out in the presence of different concentrations of S-(+)-S-312. Scatchard plot analyses of the saturation curves with \(^{3}H\)nitrendipine are shown in Fig. 5a. A decrease of the apparent dissociation constant \((K_d)\) was observed without alteration of maximal binding capacity \((B_{max})\) when the S-(+)-S-312 concentration increased from 0 \((B_{max} = 40 \text{ fmol/mg of protein}, K_d = 0.2 \text{ nM})\) to 0.3 nM \((B_{max} = 36 \text{ fmol/mg of protein}, K_d = 0.4 \text{ nM})\) and 0.7 nM \((B_{max} = 43 \text{ fmol/mg of protein}, K_d = 1.0 \text{ nM})\). Similar results were obtained with (+)-\(^{3}H\)PN200-110 binding (Fig. 5b); that is, \(B_{max}\) and \(K_d\) were 68 fmol/mg protein and 61 pM, 62 fmol/mg protein and 130 pM, and 65 fmol/mg protein and 300 pM in the presence of 0, 0.3, and 0.7 nM S-(+)-S-312, respectively.

Effect of two isomers of S-312 and other 1,4-dihydropyridines on the Ca\(^{2+}\) channel in A7r5 cells

Depolarization led to a quick and long-lasting increase in [Ca\(^{2+}\)]\(_i\) in rat aortic smooth muscle A7r5 cells. Figure 6 shows the typical traces obtained when a depolarizing concentration of KCl (70 mM) was added to A7r5 cells without or with S-(+)-S-312, nicardipine or R-(−)-S-312. S-(+)-S-312 dose-dependently suppressed the high K\(^{+}\)-induced [Ca\(^{2+}\)]\(_i\) rise, and it preferentially blocked the sustained phase rather than the initial phase (Fig. 6). This was the case with nicardipine, nisoldipine and nifedipine, but R-(−)-S-312 suppressed the sustained phases only 1.7 times more potently than the initial phase (Table 2). The rank order in inhibiting the sustained phase of the high K\(^{+}\)-induced [Ca\(^{2+}\)]\(_i\) rise was S-(+)-S-312 > nicardipine ≥ nisoldipine > R-(−)-S-312 > nifedipine.

The binding sites for 1,4-dihydropyridines in A7r5 cells were detected with (+)-\(^{3}H\)PN200-110 in the high K\(^{+}\) (70 mM) buffer. We were not able to obtain reproducible (+)-\(^{3}H\)PN200-110 binding in Heps-buffered Hanks’ solution (5 mM K\(^{+}\)). The cells in 70 mM K\(^{+}\) buffer contained a single class of specific binding sites for (+)-\(^{3}H\)PN200-110 with an apparent \(K_d\) of 39 pM and binding sites of \(1.5 \times 10^4\)/cell. As shown in Table 2, the rank order in displacing (+)-\(^{3}H\)PN200-110 binding was nisoldipine ≥ S-(+)-S-312 ≥ nicardipine > nifedipine > R-(−)-S-312, which was different from the rank order for the effect on [Ca\(^{2+}\)]\(_i\).
Fig. 6. Effect of S- (+)-S-312 (SS312), nicardipine (NC) and R-(-)-S-312 (RS312) on high K⁺-induced [Ca²⁺]ᵢ increase. The traces were obtained from one experiment, but are representative of three other experiments. Vehi., vehicle (dimethyl sulfoxide); HK⁺, high K⁺ (70 mM); LK⁺, low K⁺ (5 mM).

Table 2. Inhibition by 1,4-dihydropyridines of high K⁺-induced [Ca²⁺]ᵢ increases and (+)⁻[³H]PN200-110 binding in A7r5 cells

| Inhibitor     | [Ca²⁺]ᵢ (IC₅₀) | (+)⁻[³H]PN200-110 binding at 70 mM K⁺ |
|---------------|----------------|-------------------------------------|
|               | Initial        | Sustained                           | (Kᵣ)                      |
| Nifedipine    | 16 ± 1 (0.28)  | 4.8 ± 0.9 (0.11)                    | 0.95 ± 0.30 (0.20)         |
| Nisoldipine   | 3.3 ± 0.3 (1.33)| 0.86 ± 0.18 (0.60)                  | 0.12 ± 0.03 (1.75)         |
| Nicardipine   | 4.4 ± 0.6 (1.00)| 0.52 ± 0.02 (1.00)                  | 0.21 ± 0.11 (1.00)         |
| S- (+)-S-312  | 0.9 ± 0.1 (4.89)| 0.16 ± 0.04 (3.25)                  | 0.16 ± 0.05 (1.42)         |
| R- (-)-S-312  | 3.0 ± 0.0 (1.47)| 1.8 ± 0.4 (0.27)                    | 5.0 ± 1.0 (0.04)           |

IC₅₀ values were determined on maximum [Ca²⁺]ᵢ, within 30 sec (initial) and sustained [Ca²⁺]ᵢ, at 1–2 min (sustained) following the exposure of A7r5 cells to high K⁺. Kᵣ values were calculated as described in the legend of Table 1. [L] and Kᵣ were 50 pM and 39 pM, respectively. Each value represents the mean ± S.E. (n = 3). The values in parentheses represent the relative potency (nicardipine = 1.00).
in A7r5 cells.

DISCUSSION

We detected a rapid, saturable, reversible and specific binding of \(^{3}H\)nitrendipine in membranes prepared from porcine basilar blood vessels. The binding sites also seemed to recognize an isomeric conformation because the affinity of \(S-(+)-S-312\) was 26.4 times higher than that of \(R-(−)-S-312\). The membranes contained a single class of specific binding sites for \(^{3}H\)nitrendipine with an apparent \(K_d\) of 0.32 nM, in agreement with those obtained with membranes from other tissues such as the aorta, heart and cerebral cortex. Furthermore, there was no essential difference in \(K_c\) values among these tissues for the five blockers tested. These results suggest that the 1,4-dihydropyridine binding sites identified in basilar blood vessels possess characteristics similar to those in other tissues. We obtained similar results with another radio-labeled 1,4-dihydropyridine, \((+)-^{3}H\)PN200-110, which bound to the sites with one order of magnitude higher affinity than \(^{3}H\)nitrendipine.

S-312 has been reported to be a potent new calcium antagonist possessing vasculoselectivity, essentially for cerebral vessels (2). Although S-312 belongs to a new class of potent molecules (1,4-dihydropyridine with a fused thiophene nucleus), it and its optical isomers, \(S-(+)-S-312\) and \(R-(−)-S-312\), completely inhibited \(^{3}H\)nitrendipine or \((+)-^{3}H\)PN200-110 binding with a Hill coefficient of about 1.0. Furthermore, Scatchard analysis revealed that \(S-(+)-S-312\) influenced the affinity of both bindings with no change in the maximal density of the binding sites \((B_{max})\). This apparent competitive type of inhibition indicates that \(S-(+)-S-312\) interacts with the same receptor sites as those for nitrendipine or \((+)-^{3}H\)PN200-110.

While S-312 was 1.5 times less potent than nicardipine in inhibiting \(^{3}H\)nitrendipine binding, it has been reported to be 2 times more potent than nicardipine in the vasodilating effect on the high \(K^+\)-depolarized basilar artery (2). Furthermore, whereas \(S-(+)-S-312\) was as potent as nicardipine and nisoldipine in inhibiting labeled 1,4-dihydropyridine bindings, \(S-(+)-S-312\) inhibited the depolarization-induced \([Ca^{2+}]_i\), increase in PC12 cells more prominently than nicardipine and nisoldipine (15). To directly compare the functional and binding potencies, we used rat aortic smooth muscle A7r5 cells.

High \(K^+\) induced a small but significant increase in \([Ca^{2+}]_i\) in A7r5 cells, which was sustained for at least 2 min. This \([Ca^{2+}]_i\) increase was divided into two components by the difference in sensitivity to 1,4-dihydropyridines including \(S-(+)-S-312\) (Fig. 6). The sustained phase (more than 30 sec) was 1.7–8.5 times more sensitive to the drugs than the initial phase. As demonstrated with PC12 cells (15, 16), \((+)-^{3}H\)PN200-110 binding to intact A7r5 cells is markedly increased by \(K^+-\)depolarization. Thus, the binding sites for 1,4-dihydropyridines appear to change from a low-affinity state to a high-affinity state after depolarization of the cell membrane potential. The biphasic blockade of high \(K^+\)-induced \([Ca^{2+}]_i\) increase by submaximal concentrations of 1,4-dihydropyridines probably reflects that it takes some time for the binding to reach a new equilibrium. Next, therefore, we compared the relative potencies of the drugs for the sustained \([Ca^{2+}]_i\) and \((+)-^{3}H\)PN200-110 binding in high \(K^+\). With nicardipine as a standard, \(S-(+)-S-312\) and \(R-(−)-S-312\) inhibited the high \(K^+-\)induced \([Ca^{2+}]_i\) increase more potently than expected from their affinity for \((+)-^{3}H\)PN200-110 binding sites, while nifedipine and nisoldipine inhibited less potently. At present, we do not know why both isomers of S-312 showed more effective functional activities than expected from their affinities for 1,4-dihydropyridine binding sites. However, our results suggest that the dihydrothienopyridine derivatives may interact with the \(Ca^{2+}\) channel in a manner slightly different from the conventional 1,4-dihydropyridines.

We observed no tissue specificity of S-312
and its optical isomers as well as other 1,4-dihydropyridines in relation to the effect on \([3H]\)nitrendipine binding, although S-312 and nicardipine are reported to show selectivity for brain vessels (2, 17). Much work has suggested that the source of tissue selectivity is the extent of intracellular Ca\(^{2+}\) stores (18) and multiplicity of Ca\(^{2+}\) channel subtypes and Ca\(^{2+}\) channel states (8, 18-20). Three main states of voltage-dependent Ca\(^{2+}\) channels have been postulated: resting, open and inactivated. The state of the channel dramatically influences its affinity for 1,4-dihydropyridines (15, 16, 21). The experiments with homogenized preparations, such as membranes, could not accurately reflect the effect found in intact cells or tissues because of the loss of the original membrane potential. This might be the case with brain blood vessels.

In conclusion, the 1,4-dihydropyridine binding sites in porcine basilar blood vessels possess characteristics similar to those in other tissues described previously. S-312 and its optical isomers interact with the binding sites in a competitive manner. However, when compared with conventional 1,4-dihydropyridines, the functional activities of S-(+)-S-312 and R-(−)-S-312 were a little more potent than expected from their affinity for 1,4-dihydropyridine binding sites. Binding studies with a radiolabeled dihydrothienopyridine derivative will be required for detailed characterization for the interaction of S-(+)-S-312 or R-(−)-S-312 with the Ca\(^{2+}\) channel.

Acknowledgments
We thank Dr. Ikuo Adachi for synthesizing and supplying the Ca\(^{2+}\) channel antagonists and Drs. Motohiko Ueda, Shigeyuki Nakajima, Masatoshi Nakajima and Mitsuoshi Ninomiya of our laboratories for helpful discussions.

REFERENCES

1. Adachi, I., Yamamori, T., Hiramatsu, Y., Sakai, K., Mihara, S., Kawakami, M., Masui, M., Uno, O. and Ueda, M.: Studies on dihydropyridines. III. Synthesis of 4,7-dihydrothieno[2,3-b]-pyridines with vasodilator and antihypertensive activities. Chem. Pharm. Bull. 36, 4389–4402 (1988)

2. Ninomiya, M., Tani, T., Nakajima, S. and Ueda, M.: Effects of S-312, a new calcium antagonist, on the mechanical and electrophysiological responses of isolated cardiovascular preparations. Japan. J. Pharmacol. 51, 227–238 (1989)

3. DePover, A., Matlib, M.A., Lee, S.W., Dube, G.P., Grupp, I.L., Grupp, G. and Schwartz, A.: Specific binding of \([3H]\)nitrendipine to membranes from coronary arteries and heart in relation to pharmacological effects. Paradoxical stimulation by diltiazem. Biochem. Biophys. Res. Commun. 108, 110–117 (1982)

4. Williams, L.T. and Tremble, P.: Binding of a calcium antagonist, \([3H]\)nitrendipine, to high affinity sites in bovine aortic smooth muscle and canine cardiac membranes. J. Clin. Invest. 70, 209–212 (1982)

5. Sarmiento, J.G., Janis, R.A., Katz, A.M. and Triggle, D.J.: Comparison of high affinity binding of calcium channel blocking drugs to vascular smooth muscle and cardiac sarcolemmal membranes. Biochem. Pharmacol. 33, 3119–3123 (1984)

6. Wei, X.Y., Luchowski, E.M., Rutledge, A., Su, C.M. and Triggle, D.J.: Pharmacologic and radioligand binding analysis of the actions of 1,4-dihydropyridine activator-antagonist pairs in smooth muscle. J. Pharmacol. Exp. Ther. 239, 144–153 (1986)

7. Yamada, S., Harada, Y. and Nakayama, K.: Characterization of calcium channel antagonist binding sites labeled by \([3H]\)nitrendipine in porcine coronary artery and aorta. Eur. J. Pharmacol. 154, 203–208 (1988)

8. Morel, N. and Godfraind, T.: Pharmacological properties of voltage-dependent calcium channels in functional microvessels isolated from rat brain. Naunyn Schmiedebergs Arch. Pharmacol. 340, 442–451 (1989)

9. Mihara, S., Doteuchi, M., Hara, S., Ueda, M., Ide, M., Fujimoto, M. and Okabayashi, T.: Characterization of \([3H]\)U46619 binding in pig aorta smooth muscle membranes. Eur. J. Pharmacol. 151, 59–65 (1988)

10. Gould, R.J., Murphy, K.M.M. and Snyder, S.H.: \([3H]\)Nitrendipine-labeled calcium channels discriminate inorganic calcium agonists and antagonists. Proc. Natl. Acad. Sci. U.S.A. 79, 3656–3660 (1982)

11. Mihara, S., Shigeri, Y. and Fujimoto, M.: Neuropeptide Y-induced intracellular Ca\(^{2+}\) increases in vascular smooth muscle cells. FEBS Lett. 259, 79–82 (1989)
12 Ehlert, F.J., Itoga, E., Roeske, W.R. and Yamamura, H.I.: The interaction of [3H]nitrendipine with receptors for calcium antagonists in the cerebral cortex and heart of rats. Biochem. Biophys. Res. Commun. 104, 937–943 (1982)

13 Yamamura, H.I., Shoemaker, H., Boles, R.G. and Roeske, W.R.: Diltiazem enhancement of [3H]nitrendipine binding to calcium channel associated drug receptor sites in rat brain synaptosomes. Biochem. Biophys. Res. Commun. 108, 640–646 (1982)

14 Bolger, C.T., Gengo, P., Klockowski, R., Luchowski, E., Siegel, H., Janis, R.A., Triggle, A.M. and Triggle, D.J.: Characterization of binding of the Ca channel antagonist, [3H]nitrendipine, to guinea pig ileal smooth muscle. J. Pharmacol. Exp. Ther. 225, 291–309 (1983)

15 Fujimoto, M. and Mihara, S.: Two states of the L-type Ca2+ channel in PC12 cells: Different sensitivity to 1,4-dihydropyridines. Neurosci. Lett. 122, 9–12 (1991)

16 Greenberg, D.A., Carpenter, C.L. and Messing, R.O.: Depolarization-dependent binding of the calcium channel antagonist, (+)-[3H]PN200-110, to intact cultured PC12 cells. J. Pharmacol. Exp. Ther. 238, 1021–1027 (1986)

17 Sorkin, E.M. and Clissold, S.P.: Nicardipine: A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy, in the treatment of angina pectoris, hypertension and related cardiovascular disorders. Drugs 33, 296–345 (1987)

18 Godfraind, T., Miller, R.C. and Wibo, M.: Calcium antagonism and calcium entry blockade. Pharmacol. Rev. 38, 321–416 (1986)

19 Bean, B.P.: Nitrendipine block of cardiac calcium channels: high-affinity binding to the inactivated state. Proc. Natl. Acad. Sci. U.S.A. 81, 6388–6392 (1984)

20 Kamp, T.J., Sanguinetti, M.C. and Miller, R.J.: Voltage-dependent binding of dihydropyridine calcium channel blockers to guinea pig ventricular myocytes. J. Pharmacol. Exp. Ther. 247, 1240–1247 (1988)

21 Godfraind, T., Morel, N. and Wibo, M.: Tissue specificity of dihydropyridine-type calcium antagonists in human isolated tissues. Trends Pharmacol. Sci. 9, 37–39 (1988)