Calcium Channel Blocking Properties of SM-6586 in Rat Heart and Brain as Assessed by Radioligand Binding Assay

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ABSTRACT—The interaction of SM-6586 (methyl 1,4-dihydro-2,6-dimethyl-3-{3-(N-benzyl-N-methylaminomethyl)-1,2,4-oxadiazolyl-5-yl}-4-(3-nitrophenyl)pyridine-5-carboxylate) with the specific binding of ³H-PN200-110 to rat heart and brain membranes was characterized and compared with those of other Ca²⁺ antagonists. The blockade of ³H-PN200-110 binding sites induced by nifedipine, nitrendipine and nimodipine was reversed by washing, whereas the blockade by SM-6586 was not readily reversed under these conditions. No significant difference was found in irreversibility between SM-6586 enantiomers. When rat aortic strips were pretreated with SM-6586, the contractions induced by 50 mM KCl were inhibited even though SM-6586 was not present in the extracellular medium. This residual inhibitory effect was much stronger than that of nicardipine. The inhibition of KCl-induced contractions by nifedipine and nitrendipine was easily reversed by washing. Thus, we suggest that (+)SM-6586 is a novel 1,4-dihydropyridine derivative having a very slow rate of dissociation from the binding site. This property may explain its long-lasting antihypertensive effect.

Keywords: SM-6586, ³H-PN200-110 binding, Tissue (rat), Ca²⁺ channel, Ca²⁺ antagonistic effect

We have previously shown that a novel 1,4-dihydropyridine derivative, SM-6586 (methyl 1,4-dihydro-2,6-dimethyl-3-{3-(N-benzyl-N-methylaminomethyl)-1,2,4-oxadiazolyl-5-yl}-4-(3-nitrophenyl)pyridine-5-carboxylate), is as potent as nicardipine, nifedipine, nimodipine and nitrendipine at displacing the specific bindings of ³H-nitrendipine and ³H-PN200-110 to rat brain and heart membranes (1). In experiments with conscious spontaneously hypertensive rats (SHR), SM-6586 has been found to have a long-lasting antihypertensive effect (2, 3). Ligand binding studies have demonstrated that the long-acting dihydropyridine Ca²⁺ antagonists strongly bind to the dihydropyridine binding sites in Ca²⁺ channels and dissociate slowly from these sites (4–6). Thus, the long-lasting effect of SM-6586 may be attributable to its slow dissociation. To clarify this possibility, we examined the inhibitory effect of SM-6586 observed after its removal on ³H-PN200-110 binding to rat brain and heart membranes and on KCl-induced contractions in rat aorta in comparison with those of other Ca²⁺ antagonists.

MATERIALS AND METHODS

Radioligands
³H-PN200-110 (87 Ci/mmol) was purchased from New England Nuclear/Du pont, Inc., Boston, MA, USA.

Membrane preparation
Membrane-enriched fractions were prepared by the method described previously (1). In brief, hearts and brains were removed from Wistar rats weighing about 200–300 g and then minced in 10 mM Tris-HCl containing 250 mM sucrose (pH 7.4). The brain was gently homogenized by a glass homogenizer, and the heart was homogenized by a Polytron, twice for 10 sec at setting 8. Both homogenates were filtered through 4 layers of gauze, and the filtrates were centrifuged at 40,000 x g for 30 min. The resultant pellets were immediately rinsed with incubation medium containing 120 mM Tris-HCl buffer and 40 mM MgCl₂, pH 7.2, and then homogenized in the same medium with a glass (brain) or Polytron (heart) homogenizer. Protein was determined by the method of Lowry et al. (7).
Binding assay

The membranes were incubated with various concentrations of \(^{3}H\)-PN200-110 in a total volume of 0.5 ml of 120 mM Tris-HCl buffer and 20 mM MgCl\(_2\) (pH 7.2) at 23°C. After the incubation, the membranes were rapidly vacuum-filtered through a GF/C glass fiber filter. The tissue-bound radioactivity was counted with a scintillation counter. The specific binding was defined as the difference in binding determined in the absence and presence of 0.1 mM nifedipine. The specific binding of \(^{3}H\)-PN200-110 to rat heart and brain membrane increased linearly with increasing protein concentrations in the range of 0.05–0.25 mg per assay and was at equilibrium at 40 min (data not shown). Thus, the binding reaction was carried out for 45 min, with a protein concentration of 0.2 mg (heart) and 0.1 mg (brain) per assay. The dissociation constant (K\(_d\)) and maximal binding sites (B\(_{max}\)) for heart and brain \(^{3}H\)-PN200-110 binding were estimated by Scatchard analysis of the saturation data over the concentration range of 0.01–2.5 nM and 0.01–0.8 nM \(^{3}H\)-PN200-110, respectively.

The inhibitory effects of Ca\(^{2+}\) antagonists after their removal on \(^{3}H\)-PN200-110 binding were studied according to the method described by Yamada et al. (8, 9). The membranes were preincubated with Ca\(^{2+}\) antagonists or without added antagonists for 45 min. The preincubated membranes were washed 3 times in 60 mM Tris-HCl buffer and 20 mM MgCl\(_2\) (pH 7.2) by centrifugation and resuspension. The washed membranes were then assayed for \(^{3}H\)-PN200-110 binding. The concentration of \(^{3}H\)-PN200-110 used was 0.06 nM.

Pharmacological experiments

Spirally cut strips of thoracic aortae were obtained from male Wistar rats, weighing 300–350 g, as described previously (1). After the contractile response to 50 mM KCl was determined, the preparation was incubated with a Ca\(^{2+}\) antagonist for 30 min and again was exposed to 50 mM KCl. Then, the preparation was washed three times with drug-free Krebs-Henseleit solution. Exposure of the preparation to 50 mM KCl was repeated at intervals of 30 min until 240 min after removal of the drug.

RESULTS

The representative saturation isotherms for \(^{3}H\)-PN200-110 and their Scatchard plots are shown in Fig. 1. The radioligand interacted with a single population of saturable high-affinity sites in both heart and brain membranes. The K\(_d\) value for \(^{3}H\)-PN200-110 in heart membranes was significantly higher than that in brain membranes (Table 1).

The reversibility of the inhibition of heart and brain \(^{3}H\)-PN200-110 binding by SM-6586 and other Ca\(^{2+}\) antagonists is shown in Fig. 2. In the presence of 10\(^{-8}\) M nifedipine or 10\(^{-9}\) M nitrendipine, nimodipine, nicardipine.

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**Fig. 1.** Saturation isotherms for \(^{3}H\)-PN200-110 binding to membranes from rat heart (left) and brain (right). Total (open circles), specific (solid circles) and nonspecific (solid squares) binding for \(^{3}H\)-PN200-110 were determined in parallel assays in the absence and presence of 0.1 mM nifedipine. Scatchard plot of the data is shown in each lower panel.
pine or SM-6586, about 50–70% of the specific $^3$H-PN200-110 binding to the heart and brain membranes was inhibited. When the membranes previously exposed to nifedipine, nitrendipine or nimodipine at the above concentrations for 45 min were washed extensively and subsequently assayed for $^3$H-PN200-110 binding, the specific binding was significantly restored. In contrast, there was little recovery of specific $^3$H-PN200-110 binding in nicardipine- or SM-6586-pretreated membranes. The irreversible inhibition of $^3$H-PN200-110 binding induced by the enantiomers of SM-6586 was substantially equal to that by the racemate.

Nifedipine ($3 \times 10^{-9}$ M), nitrendipine ($3 \times 10^{-9}$ M), nicardipine ($3 \times 10^{-9}$ M) and SM-6586 ($6 \times 10^{-9}$ M) inhibited 50 mM KCl-induced contractions similarly by 50% in rat aortic strips. The time courses of tension recovery following the washout of these Ca$^{2+}$ antagonists are shown in Fig. 3. The KCl-induced contraction almost completely recovered 60 min after the washout of nifedipine and 150 min after the washout of nitrendipine. The time course of recovery was slower when nicardipine was used for pretreatment. When the aortic strips were pretreated with SM-6586, the aortic tension did not significantly recover after repeated washout for 240 min.

There was a good correlation between the inhibition of $^3$H-PN200-110 binding to the heart and brain membranes

| Table 1. $K_d$ and $B_{max}$ values for $^3$H-PN200-110 binding to rat heart and brain membranes |
|-----------------|-----------------|-----------------|-----------------|
|                 | $K_d$ (nM)      | $B_{max}$ (fmol/mg protein) |
| Heart           | 0.512±0.051*    | 93.13±10.49 (8)  |
| Brain           | 0.169±0.020     | 65.41±5.18 (6)   |
| Values are means±S.E. The number of experiments are indicated in parentheses. The asterisk shows a significant difference between heart and brain membranes (P<0.01).

Fig. 2. Inhibition by Ca$^{2+}$ antagonists on $^3$H-PN200-110 binding to rat heart and brain membranes. White columns: the membranes were incubated with $^3$H-PN200-110 in the presence of each Ca$^{2+}$ antagonist. Dotted columns: the membranes were preincubated with each Ca$^{2+}$ antagonist, and then they were washed as described in Materials and Methods. The washed membranes were then assayed for $^3$H-PN200-110 binding. The ordinate represents $^3$H-PN200-110 binding expressed as a percentage of the specific binding of 0.06 nM $^3$H-PN200-110 in the absence of any antagonist. The concentrations of the Ca$^{2+}$ antagonists used in these experiments are $3 \times 10^{-9}$ M (nifedipine, nitrendipine, nimodipine and nicardipine) and $6 \times 10^{-9}$ M (SM-6586). Each column represents the mean±S.E. of five experiments. *P<0.01 vs. the corresponding values before washing.

Fig. 3. Residual inhibitory effects of Ca$^{2+}$ antagonists after their washout on 50 mM KCl-induced contractions in rat aortic strips. Thirty minutes after the addition of each Ca$^{2+}$ antagonist, the strips were washed three times with normal solution and then stimulated with 50 mM KCl followed by washout. This cycle was repeated. Ordinate: % inhibition of 50 mM KCl-induced contraction. Each value is the mean±S.E. of six experiments.
and the inhibition of the KCl-induced contraction in rat aortic strips caused by the Ca\(^{2+}\) antagonists after their removal (Fig. 4).

**DISCUSSION**

We have previously reported that the potency of SM-6586 is comparable to those of nicardipine, nitrendipine, nimodipine and nifedipine in inhibiting \(^3\)H-nitrendipine binding and \(^3\)H-PN200-110 binding to rat heart and brain membranes (1). In this study, however, we found that the blockade of \(^3\)H-PN200-110 binding sites in the heart and brain induced by nifedipine, nitrendipine and nimodipine was easily reversed by washing, whereas the blockade by SM-6586 or nicardipine was not reversed under these conditions. This indicates that, in contrast to nifedipine, nitrendipine and nimodipine, once SM-6586 and nicardipine are bound to their site of action, they are not readily removed by washing. Thus, SM-6586 and nicardipine tightly bind to dihydropyridine binding sites in Ca\(^{2+}\) channels and only slowly dissociate from the sites. These properties of SM-6586 and nicardipine may be responsible for the long-lasting antihypertensive effects seen in SHR models. It seems that the rate of dissociation of SM-6586 may be slower than the rate of dissociation. The residual inhibitory effect of SM-6586 on KCl-induced contractions in rat aorta was much stronger than those of other Ca\(^{2+}\) antagonists, including nicardipine. When the aortic strips were pretreated with SM-6586, KCl-induced contractions did not significantly recover even after repeated washout for 240 min.

The importance of the chemical structures for Ca\(^{2+}\) antagonistic actions has been reported (10–12). As suggested in our previous report (1), SM-6586 is a 1,4-dihydropyridine derivative, and its important chemical structures are 1) the stereochemistry at C-4, 2) the position of the NO\(_2\) substitution of the nitrophenyl group, and 3) the \(N,N\)-benzyl methylamino substitution of the C-3 in the 1,4-dihydropyridine ring. We did not recognize a difference in irreversibility between stereoisomers of SM-6586, indicating that the stereochemistry at C-4 does not play an important role in the irreversible effect of SM-6586. The differences in the structures of nitrendipine and nifedipine are the substitution at C-5 and the position of the NO\(_2\) in the nitrophenyl group. These differences might contribute to the different dissociation rates of these antagonists from the binding sites. Nevertheless, as the dissociation rate of these antagonists appeared similarly faster than those of SM-6586 and nicardipine, the importance of these structural differences for irreversibility may be less meaningful. On the other hand, a substitution at C-3 may be crucial for the irreversibility of the Ca\(^{2+}\) antagonists. The order of the molecular size of the substitution at C-3 was SM-6586 > nicardipine > nimodipine > nitrendipine > nifedipine. Thus, the C-3 substitution of SM-6586 may affect the tight binding of this drug to Ca\(^{2+}\) channels.

In conclusion, although the Ca\(^{2+}\) antagonistic potency of SM-6586 was almost the same as those of other Ca\(^{2+}\) antagonists, its binding was the tightest among the drugs tested. These comparative studies suggest that the substitution at C-3 of the 1,4-dihydropyridine ring, rather than
the substitution at C-5 and the position of NO₂ substitution in the nitrophenol ring, may contribute to the irreversible Ca²⁺ antagonistic effect of SM-6586, leading to the long-lasting antihypertensive effect.

REFERENCES


