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Residual inhibition in density of [^{3}H]isradipine binding sites in rat brain membrane pretreated with amlodipine

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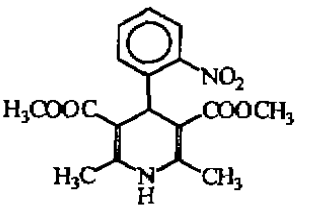
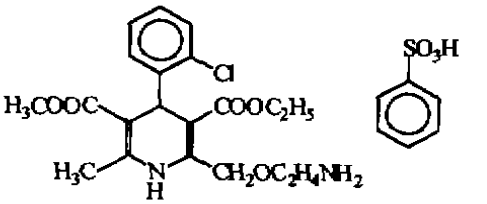
AIM: To test changes in the density of [^{3}H] isradipine binding sites in rat brain membrane pretreated with amlodipine and to compare with those of nifedipine and (+) SM-6586 (methyl 1, 4-dihydro-2, 6-dimethyl-3-(3-(*N*-benzyl-*N*-methylaminomethyl)-1, 2, 4-oxadiazolyl-5-yl)-4-(3-nitrophenyl) pyridine-5-carboxylate). **METHODS**: The membrane-enriched fractions were prepared from rat brain. The brain membranes were preincubated with nifedipine (10 nmol L^{-1}), amlodipine (1 μmol L^{-1}) and SM-6586 (1 nmol L^{-1}) or with no antagonists added for 45 min, and washing and centrifugation were performed 3 times. They were assayed with [^{3}H]isradipine in incubation media. The K_d and B_{max} values of the membrane fractions pretreated with the drugs were determined by Scatchard analysis. **RESULTS**: The blockage of the [^{3}H]isradipine binding sites induced by nifedipine was reversed by washing, enabling the low values of the specific binding sites to be observed. The blockages by amlodipine and SM-6586, on the other hand, were not readily reversed. No significant difference was found, however, between in the K_d walues of these drugs. **CONCLUSION**: Amlodipine and SM-6586 are Ca^{2+} antagonists which dissociate slowly from the Ca^{2+} channel in membranes.

KEY WORDS amlodipine; isradipine; nifedipine; SM-6586; calcium channel blockers; brain; radioligand assay

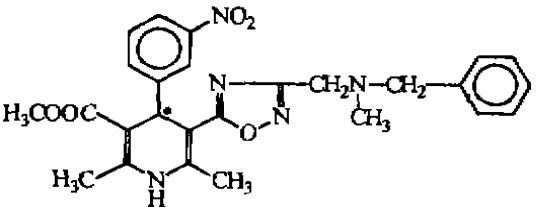
Amlodipine exhibited slow rates of association and dissociation with respect to [^{3}H]isradipine binding sites in rat brains[1-3]. Amlodipine exhibits a slow onset and a very long duration of action clinically[4]. The blockage of [^{3}H]isradipine binding sites by (+)SM-6586 (methyl 1,4-dihydro-2,6-dimethyl-3-(3-(*N*-benzyl-*N*-methylaminomethyl)-1,2,4-



Nifedipine



Amlodipine



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oxadiazolyl-5-yl)-4-(3-nitrophenyl) pyridine-5-carboxylate) was not readily reversed, unlike that of nifedipine, suggesting that this compound has a very slow rate of dissociation from the binding sites[5]. The long-lasting Ca^{2+} antagonistic effects of amlodipine and SM-6586 may be due to this slow onset of their association with and dissociation from 1, 4-dihydropyridine (DHP) receptors[6], implying that these 2 compounds may bind tightly with the receptor sites.

The present study was to examine the residual inhibition effects of nifedipine, amlodipine, and SM-6586 on [^{3}H] isradipine bindings in rat brain membranes pretreated with these Ca^{2+} antagonists and after washing with buffers.

MATERIALS AND METHODS

Preparation of membrane-enriched fractions from rat brain Membrane-enriched fractions were prepared according to a method described previously[5]. The brains from ♂ Wistar rats (weighing 200—300 g) were homogenized in Tris-HCl containing sucrose (pH 7.4). The filtrates were centrifuged at 40 000 × g for 30 min. The pellets were rinsed with incubation medium containing Tris-HCl buffer and $MgCl_2$ (pH 7.2), then gently homogenized. The homogenates were used for the radioligand assay. Protein content was determined colorimetrically[7].

Radioligand assay The inhibitory effects of nifedipine, amlodipine, and (+)SM-6586 on [^{3}H]isradipine binding after their removal were determined[5]. The membranes were preincubated at 23 ℃ with nifedipine (10 nmol L^{-1}), amlodipine (1 μmol L^{-1}) and (+)SM-6586 (1 nmol L^{-1}) or with no added antagonist for 45 min, using 0.1 mg protein concentration of brain membrane fractions. The drug concentration used showed an almost 50 % inhibition by the displacemental experiment. The preincubated membranes were washed 3 times in Tris-HCl 60 mmol L^{-1} buffer and $MgCl_2$ 20 mmol L^{-1} (pH 7.2) by centrifugation and resuspension. The washed membranes were incubated with [^{3}H]isradipine 0.08—1.67 nmol L^{-1} in a total volume of 0.5 mL of Tris-HCl 120 mmol L^{-1} buffer and $MgCl_2$ 20 mmol L^{-1} (pH 7.2) at 23 ℃ for 45 min. The membranes were vacuum-filtered through a GF/C glass fiber filter, and the tissue-bound radioactivity was counted with a scintillation counter. The specific binding of the control was defined as the difference in binding determined in the absence and presence of nifedipine 0.01 nmol L^{-1}.

All kinetic analyses were carried out on an NEC PC-9801 computer system that performed iterative linear regression, and the K_i values of the drugs were calculated[5].

Radioligand and drugs [^{3}H]Isradipine (3219 TBq mol^{-1}) was purchased from New England Nuclear/DuPont, Boston MA. USA. Amlodipine and SM-6586 were kindly donated by Sumitomo Pharmaceuticals Co Japan.

The values of the dissociation constant (K_d) and the maximal binding site (B_{max}) were calculated using Scatchard analysis.

RESULTS

The pK_i values ($\bar{x}\pm s$) were found to be nifedipine 8.3±0.7 (n=7), amlodipine 9.0±0.3 (n=4), and SM 7.4±0.2 (n=4), respectively.

[^{3}H]Isradipine bindings to membranes without pretreatmint with Ca^{2+} antagonist were saturated in the concentrations of the radioligand 0.1—1.5 mmol L^{-1} and the apparent specific bindings of the radioligand were seen. Although only small differences were seen between the densities of the total and nonspecific bindings for [^{3}H]isradipine in membranes pretreated with nifedipine, clear differences were observed between those for [^{3}H]isradipine in membranes pretreated with amlodipine and SM-6586 (Fig 1).

The data shown in Fig 1 were determined using the Scatchard analysis (Fig 2 and Tab 1).

The Scatchard analysis was linear in untreated membranes and those treated with Ca^{2+} antagonists. The K_d and B_{max} values in the rat brain with no calcium antagonist

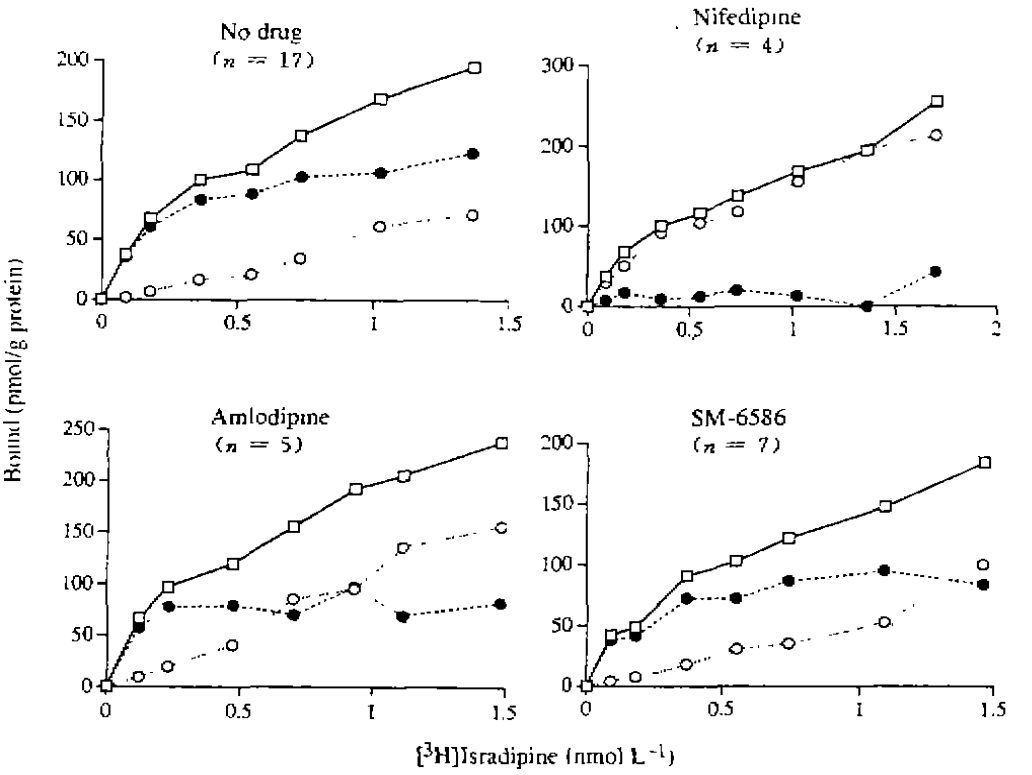

Fig 1. Saturation experiment for binding of [^{3}H]isradipine to rat brain membranes treated with nifedipine, amlodipine, or SM-6586. Only left upper plot includes nifedipine 100 mmol L^{-1}. The specific binding of [^{3}H]isradipine (●) was defined as the difference between the total (□) and the nonspecific binding (○) in the presence of nifedipine, amlodipine or SM-6586 bound membranes.

Tab 1. K_d and B_{max} in Ca^{2+} channels of rat brain membranes after washing. $\bar{x}\pm s$.
$^aP>0.05$, $^bP<0.01$ *vs* Control.

Drug	nmol L^{-1}	n	K_d (nmol L^{-1})	B_{max} (pmol/g protein)
Control	0	17	0.66±0.37	177±45
Nifedipine	10	4	0.33±0.20^a	19.2±2.8^b
Amlodipine	1 000	5	0.45±0.29^a	109±32^b
SM-6586	1	7	0.73±0.74^a	115±55^b

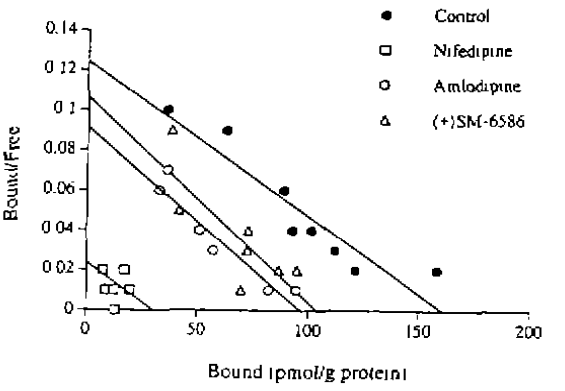

Fig 2. Scatchard plots from the data in Fig 1.

treatment were found to be 0.66±0.37 and 177±45, respectively. Although no differences in K_d values were found in the membranes treated with nifedipine, amlodipine, or SM-6586, a reduction of B_{max} values occurred in membranes treated with amlodipine and SM-6586. The B_{max} value in membranes receiving nifedipine treatment, on the other hand, was 19.2±2.8.

DISCUSSION

Amlodipine manifests Ca^{2+} antagonistic action of an extremely long duration[1-3]. SM-6586 is a long-acting Ca^{2+} antagonist[5,9]. Unlike nifedipine, these drugs display very slow association and dissociation with the Ca^{2+} channel. Thus, it was of interest to test the dissociating potencies of Ca^{2+} antagonists from the binding sites, because the prolonged hypotensive effects of these drugs may be partly due to the slow rate of dissociation from the receptor sites.

In a previous study[5], we reported a radioligand assay method to assess the potency of the dissociation of Ca^{2+} antagonists from [^{3}H]isradipine binding sites in the membranes of rat hearts and brains. In the present study, no changes were noted in the K_d values from the membranes with or without the drugs, but B_{max} values were changed, implying that the binding characteristics (dissociation constant; K_d values) of [^{3}H]isradipine to membranes pretreated with the drugs were not changed. These results suggest that numbers of [^{3}H]isradipine bound to membranes after treatment with the drugs were quantitatively observed.

The present study succeeded in quantitatively determining the residual inhibitory effects by Ca^{2+} antagonists for densities (B_{max} values) of [^{3}H]isradipine bindings in membranes pretreated with these compounds using Scatchard analysis. This method is useful if the binding is tight at rat brain membranes. It is possible that both amlodipine and SM-6586 can bind receptors and that neither can be easily dissociate from the sites, in contrast to nifedipine which is easily removed from the binding sites, the B_{max} value treated with nifedipine was very low after washed membranes by buffers. The slow dissociation of amlodipine and SM-6586 from the binding sites contributes to the long duration of the Ca^{2+} antagonistic effects. The present study showed no difference between the rate of dissociation from the binding sites in membranes for amlodipine and SM-6586. Nifedipine, on the other hand showed a difference from these 2 compounds. The importance of the chemical structure for the Ca^{2+} antagonistic effects of amlodipine has been reported[8]. Our previous report[9] also indicated that (1) the position of the NO_2 substitution of the nitrophenyl group, (2) the methylamino substitution, (3) the molecular size of the C-3 substitution, and (4) the stereo chemistry at C-4 of the 1,4-DHP ring were all important for these effects. The chemical structures of these 1,4-DHP derivatives differ mainly in the substituents of C-2 and C-3 in the DHP ring and/or in the NO_2 or chloride substitution of the nitrophenyl group. Amlodipine and SM-6586 are different in regard to the positions of these substituents at the C-2 or C-3 position in the DHP ring, and in the chloride substitution of the NO_2 group. As the potencies of the rates of dissociation from the binding sites of these 2 compounds were almost identical, however the difference between nifedipine and these 2 compounds may be crucial for these effects. The different chemical structure of nifedipine has an ester bond at C-3, and this is a small methyl acetate molecule, which is different from those of amlodipine and SM-6586. Thus, in the molecular structure, the molecular size at C-3 or C-2 of the DHP ring, in addition to the position of NO_2 in the nitrophenyl ring, may be responsible in part for the long duration of the Ca^{2+} antagonistic effects[10].

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氨氯地平抑制大鼠脑膜与[^{3}H]伊拉地平结合的密度

R965.2

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目的：研究硝苯地平、氨氯地平和(+)SM-6586预处理过的大鼠脑膜与[^{3}H]伊拉地平观察结合密度的变化。**方法**：用大鼠脑膜，该脑浆膜与硝苯地平(10 nmol L^{-1})，氨氯地平(1 μmol L^{-1})，SM-6586 (1 nmol L^{-1})以及不加拮抗剂分别预保温45 min，离心，所得沉淀用缓冲液清洗，3次后与[^{3}H]伊拉地平一起保温，通过 Scatchard 解析求出用钙拮抗剂预处理后膜成分的 K_d 和 B_{max} 值。**结果**：硝苯地平对[^{3}H]伊拉地平取代位置的拮抗可以通过清洗而减弱，特殊取代显示出很低的值。但通过氨氯地平和 SM-6586拮抗后，在同样条件下拮抗不易被减弱。以上这些药物的 K_d 值都没有明显的变化。**结论**：氨氯地平和 SM-6586对脑膜中钙通道拮抗的分解过程是缓慢的。

关键词 氨氯地平；伊拉地平；硝苯地平；SM-6586；钙通道阻滞剂；脑；放射配位体测定