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Calcium Antagonism by KB-2796, a New Diphenylpiperazine Analogue, in Dog Vascular Smooth Muscle

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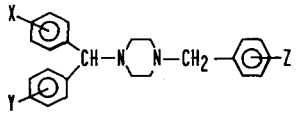
Abstract—The effects of KB-2796, a new diphenylpiperazine analogue, on [^{3}H]nitrendipine ([^{3}H]NTD) binding, KCl-induced contraction and ^{45}Ca influx has been examined in dog vascular smooth muscle, and compared with those of other diphenylpiperazines. In the binding study, [^{3}H]NTD was found to bind with a high affinity to a single class of sites on aortic membranes ($K_d = 0{\cdot}41$ nM and $B_{max} = 31$ fmol (mg protein)$^{-1}$). KB-2796 inhibited specific [^{3}H]NTD binding in a concentration-dependent manner, with a K_i value of 0·34 μM. The other diphenylpiperazine derivatives such as flunarizine and cinnarizine also inhibited binding in the same manner. Also, in the contraction study, all the diphenylpiperazines antagonized the 50 mM KCl-induced contraction of isolated mesenteric arteries concentration-dependently. The IC50 values of the compounds for KCl-induced contraction correlated strongly with the respective K_i values obtained in the [^{3}H]NTD binding study. In the ^{45}Ca influx study, KB-2796 also effectively inhibited KCl-induced ^{45}Ca influx in mesenteric arteries, with an IC50 value of 0·14 μM. This was close to the IC50 value found in the KCl-induced contraction study. These findings suggest that calcium antagonism by KB-2796 is responsible for its vasorelaxing action in vascular smooth muscle.

Voltage-dependent calcium channels regulate a variety of biological processes including muscle contraction, hormone secretion and neurotransmitter release and are inhibited by calcium antagonists which are important both as therapeutic drugs and as experimental tools (Godfraind et al 1986; Vanhoutte 1987; Nayler 1988). Currently-used calcium antagonists are structurally heterogeneous organic compounds, and are generally classified into four major groups: (1) 1,4-dihydropyridines, (2) phenylalkylamines, (3) benzothiazepines and (4) diphenylpiperazines (Godfraind et al 1986).

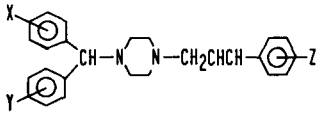
KB-2796, 1-[bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl)piperazine dihydrochloride (Fig. 1), is a newly-synthesized, diphenylpiperazine-type calcium antagonist (Ohtaka et al 1987). It has been reported that KB-2796 inhibits the contraction of dog isolated arteries induced by K^+ and by prostaglandin $F_{2\alpha}$ (Kanazawa & Toda 1987), and selectively increases cerebral blood flow in anaesthetized dogs (Kanazawa et al 1990). In addition, we showed that KB-2796 inhibits [^{3}H]nitrendipine ([^{3}H]NTD) binding to guinea-pig cerebral cortex membranes (Iwamoto et al 1988b).

The existence of high affinity binding sites of [^{3}H]NTD to vascular smooth muscle membranes has been demonstrated by several investigators (Triggle et al 1982; Williams & Tremble 1982; Pinquier et al 1988). The functional significance of this site is indicated by the fact that there is a quantitative correlation between vasorelaxation and dihydropyridine-induced inhibition of [^{3}H]NTD binding (Bolger et al 1983; Janis et al 1984; Goll et al 1986).

The purpose of this study was to determine the interaction of KB-2796 with [^{3}H]NTD binding in vascular smooth muscle and to clarify whether its inhibitory binding activity is responsible for its vasorelaxing action. We also evaluated the effects of KB-2796 on binding of [^{3}H]NTD to dog aortic membranes, and on high KCl-induced contraction and ^{45}Ca influx in dog arteries. Furthermore, we investigated the effects of some newly synthesized diphenylpiperazine derivatives, whose chemical structures are shown in Fig. 1. A preliminary report of these findings has been made in abstract form (Iwamoto et al 1988a).



	X	Y	Z
KB-2796	4-F	4-F	2,3,4-OCH_3
Compound A	3-OH	4-Cl	3-CH_3
Compound B	4-OCH_3	4-OCH_3	2,3,4-OCH_3



	X	Y	Z
Flunarizine	4-F	4-F	
Cinnarizine			
Compound C	4-F	4-F	2,3,4-OCH_3

FIG. 1. The structures of KB-2796 and other diphenylpiperazines.

Materials and Methods

Drugs

KB-2796 and diphenylpiperazine derivatives (compounds A, B and C) were synthesized by Dr H. Ohtaka of the New Drug Research Laboratories, Kanebo Ltd, Flunarizine, cinnarizine, nifedipine and nicardipine were purchased from Sigma (St. Louis, MO, USA). [5-Methyl-^{3}H]NTD (3·0 TBq

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mmol^{-2}) and $^{45}CaCl_2$ (0·4–1·5 GBq mg^{-1}) were obtained from New England Nuclear and Amersham, Japan, respectively.

For binding studies, diphenylpiperazines were dissolved in a 0·1 M HCl solution containing 50% (v/v) ethanol, and nifedipine and nicardipine were dissolved in 99·5% (v/v) ethanol. All drugs for contraction and ^{45}Ca influx studies were dissolved in 99% (v/v) dimethyl sulphoxide (DMSO) and 99·5% (v/v) ethanol, respectively. These stock solutions were diluted with distilled water to the desired concentrations. The final concentrations of ethanol and DMSO in the incubation solution were 0·5% (v/v) and 0·1% (v/v), respectively, at maximum.

Membrane preparation

Mongrel dogs of either sex (10–20 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and killed by bleeding from the common carotid arteries. The thoracic aorta was removed and the intima and adventitia were carefully dissected away from the blood vessels. The cleaned tissues were cut into small cubes and then homogenized with a Physcotron homogenizer in 7 vol of ice-cold 50 mM Tris-HCl buffer containing 0·25 M sucrose and 10 mM $MgCl_2$ (pH 7·4). The homogenate was centrifuged at 500 *g* for 10 min at 4°C. The pellets were discarded and the supernatant was filtered through three layers of cheesecloth and centrifuged at 10000 *g* for 10 min at 4°C. The resulting supernatant was further centrifuged at 105000 *g* for 30 min at 4°C. The final pellet was resuspended in ice-cold 50 mM Tris-HCl buffer. The membrane fraction was stored at −70°C for up to one week. The concentration of protein was determined by the method of Lowry et al (1951) using bovine serum albumin as the standard.

[^{3}H]NTD binding assay

Vascular membranes (500 μg protein) were incubated for 60 min at 25°C in a total volume of 2 mL of 50 mM Tris-HCl buffer (pH 7·4) with various concentrations of [^{3}H]NTD (0·05–5 nM) and drugs. After incubation in a dark tube, membranes with bound [^{3}H]NTD were trapped over Whatman glass filters (GF/C) and the precipitates were washed three times with 5 mL amounts of ice-cold buffer. The filters were placed in scintillation vials with 10 mL of scintillant for at least 6 h and the radioactivity was counted with a Packard liquid scintillation counter at an efficiency of about 50%. [^{3}H]NTD binding in the presence of 5 μM nifedipine was defined as non-specific binding and was subtracted from the total binding to obtain specific binding. Non-specific binding accounted for 50–70% of the total binding.

Preparation of arterial strips

Mongrel dogs were killed as described above. The mesenteric arteries (0·5–1·0 mm outer diameter) and cerebral arteries (basilar and middle cerebral arteries, 0·5–1·0 mm in outer diameter) were excised, cleaned of connective tissue and cut into spiral strips approximately 20 mm long. These strips were attached to stainless holders in organ baths containing physiological salt solution of the following composition (mM): NaCl 120, KCl 5·4, $NaHCO_3$ 25, $CaCl_2$ 2·2, $MgCl_2$ 1·0 and glucose 5·6 (pH 7·3–7·4). This solution was aerated continuously with a mixture of 95% O_2-5% CO_2 and kept at 37 ± 0·5°C. For the measurement of tension, a stainless steel holder anchoring the upper end of the strip was connected to a lever of a force-displacement transducer (Nihon Kohden, Tokyo, Japan) under resting tension of 1·5 g, which is optimal for inducing maximum contractions (Toda et al 1978). Before the start of the experiments, each strip was allowed to equilibrate in the physiological salt solution for 60 to 90 min.

Contraction measurements

Isometric contractions and relaxations were recorded. Mesenteric arteries were contracted with 50 mM KCl in the physiological salt solution. After the contraction had reached a plateau, drugs were added directly to the bath cumulatively at 20–30 min intervals. At the end of each series of experiments, papaverine (100 μM) was added to the bath to obtain the maximum relaxation.

^{45}Ca influx measurements

^{45}Ca influx was determined by a 'lanthanum method' (Karaki & Weiss 1979) with some modifications. Arterial strips weighing about 15 mg were incubated for 5 min in a ^{45}Ca-containing normal physiological salt solution (19 MBq mL^{-1}) to allow exchange of extracellular calcium with the tracer. The strips were incubated for another 5 min in the ^{45}Ca-containing physiological salt solution in the presence or absence of 50 mM KCl. In parallel strips, KB-2796 (0·01, 0·1 or 1 μM) was given for 30 min before the addition of 50 mM KCl. In another group of experiments, some strips were incubated for 30 min in the ^{45}Ca-containing physiological salt solution to determine the non-stimulated ^{45}Ca influx. Thereafter, the strips were soaked for 60 min in an ice-cold lanthanum solution of the following composition (mM): $LaCl_3$ 80·8, glucose 11 and Tris (hydroxymethyl)aminomethane 6·0 (pH 6·8 with maleic acid). The strips were then weighed, placed in scintillation vials and digested with a tissue solubilizer (Soluene-350, Packard) at 50°C for 3 h. Radioactivity remaining in the tissue was counted using a Packard liquid scintillation counter. ^{45}Ca influx (nmol (g wet wt tissue)$^{-1}$) was calculated as the product of the ^{45}Ca tissue/medium ratio and the total Ca^{2+} concentration in the bathing solution. The ^{45}Ca influx induced by the stimulant was computed as the difference between the influx in the presence of the stimulant and the corresponding control.

Data analysis

Concentrations producing 50% inhibition (IC50) of [^{3}H]NTD binding and KCl-induced contraction were determined using linear regression analysis on a personal computer (PC-9801, NEC). Inhibition constants (K_i) were calculated from the equation of Cheng & Prusoff (1973): $K_i = IC50/(1 + L/K_d)$, where L is the concentration of ligand.

Results

[^{3}H]NTD binding to dog aortic membranes

The binding of [^{3}H]NTD to dog aortic membranes at 25°C was saturable and specific. Scatchard analysis of the saturation isotherms was consistent with an interaction at a single population of binding sites with a dissociation constant (K_d)

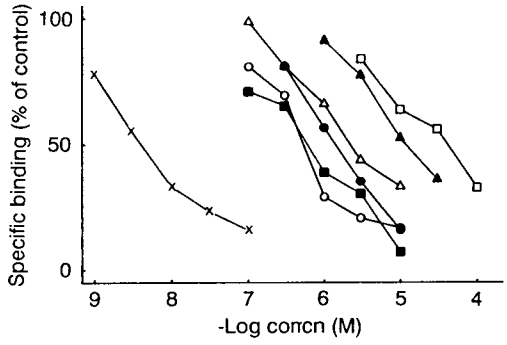

FIG. 2. Inhibition of specific [^{3}H]NTD binding to dog aortic membranes by diphenylpiperazines and nicardipine. Membranes (500 μg protein/tube) from dog aorta were incubated with 0·5 nM [^{3}H]NTD in the presence of various concentrations of drugs for 60 min at 25°C. The data are the means of three or four experiments, each performed in duplicate. KB-2796 ○, flunarizine ●, cinnarizine △, compound A ▲, compound B □, compound C ■, nicardipine X.

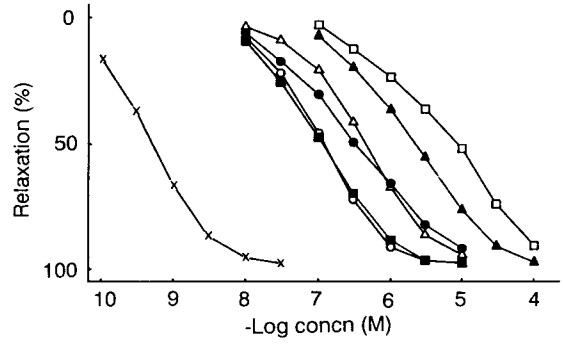

FIG. 3. Inhibition of KCl-induced contraction in dog mesenteric arteries by diphenylpiperazines and nicardipine. Relaxations induced by 100 μM papaverine were taken as 100%. The data are the means of three or four experiments. KB-2796 ○, flunarizine ●, cinnarizine △, compound A ▲, compound B □, compound C ■, nicardipine X.

of 0·41 ± 0·17 nM, and a maximal number of binding sites (B_{max}) of 31 ± 9 fmol (mg protein)$^{-1}$ (n = 3).

Effects of KB-2796 and other diphenylpiperazines on [^{3}H]NTD binding

The specific binding of [^{3}H]NTD to dog aortic membranes was inhibited in a concentration-dependent manner by all the diphenylpiperazine derivatives tested (Fig. 2). The K_i values of all the diphenylpiperazines are summarized in Table 1. The inhibitory activity of KB-2796 equalled that of compound C, and was more potent than those of flunarizine, cinnarizine, compound A and compound B. Nicardipine, a 1,4-dihydropyridine-type calcium antagonist, was the most potent inhibitor of [^{3}H]NTD binding among the calcium antagonists tested.

Vasorelaxing effects of KB-2796 and other diphenylpiperazines

To evaluate the functional significance of drug interactions with [^{3}H]NTD binding, we determined the ability of KB-2796 and other diphenylpiperazines to inhibit 50 mM KCl-induced contraction in dog mesenteric arteries. All the drugs produced concentration-dependent inhibition of the 50 mM KCl-induced contraction (Fig. 3). The IC50 values calculated from inhibition curves are listed in Table 1. The rank order of relaxing potency was as follows: KB-2796 > compound C > flunarizine > cinnarizine > compound A > compound B. The K_i values of the diphenylpiperazines for inhibition of [^{3}H]NTD binding were strongly correlated with the IC50 values for inhibition of 50 mM KCl-induced contraction (r = 0·95, Fig. 4).

Table 1. Effects of diphenylpiperazines and nicardipine on [^{3}H]NTD binding in dog aorta and on KCl-induced contraction in dog mesenteric arteries.

Compound	[^{3}H]NTD binding K_i (μM)	Contraction IC50 (μM)
KB-2796	0·34 ± 0·1	0·12 ± 0·02
Flunarizine	0·68 ± 0·05	0·43 ± 0·2
Cinnarizine	1·3 ± 0·2	0·45 ± 0·08
Compound A	19 ± 12	2·0 ± 0·8
Compound B	16 ± 6	7·6 ± 4
Compound C	0·26 ± 0·1	0·13 ± 0·04
Nicardipine	0·003 ± 0·001	0·0005 ± 0·00006

The data are the means ± s.e.m. of either three or four experiments.

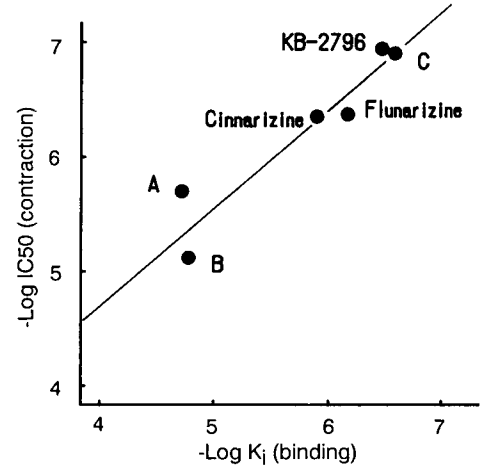

FIG. 4. Relationship between the inhibition (K_i) of [^{3}H]NTD binding in dog aorta by diphenylpiperazines and their pharmacological activities (IC50 values for the inhibition of 50 mM KCl-induced contraction) in dog mesenteric arteries (Table 1). The correlation coefficient is r = 0·95 ($P < 0·01$).

Effects of KB-2796 on ^{45}Ca influx

The effect of KB-2796 on the activity of voltage-dependent calcium channels was investigated by ^{45}Ca influx experiments on dog cerebral and mesenteric arteries. KB-2796 (0·01–1 μM) inhibited the ^{45}Ca influx component elicited by 50 mM KCl in dog cerebral and mesenteric arteries, and this effect was concentration-dependent (Fig. 5). The calculated IC50 value of KB-2796 in dog cerebral arteries (0·06 ± 0·01 μM; n = 10) was smaller than that in mesenteric arteries (0·14 ± 0·02 μM; n = 6). In mesenteric arteries, the IC50 value of KB-2796 for ^{45}Ca influx was similar to that for 50 mM KCl-induced contraction (0·12 μM). In a physiological salt solution containing 5·4 mM KCl, however, KB-2796 (1 μM) produced no significant effect on the resting ^{45}Ca influx in both arteries (data not shown).

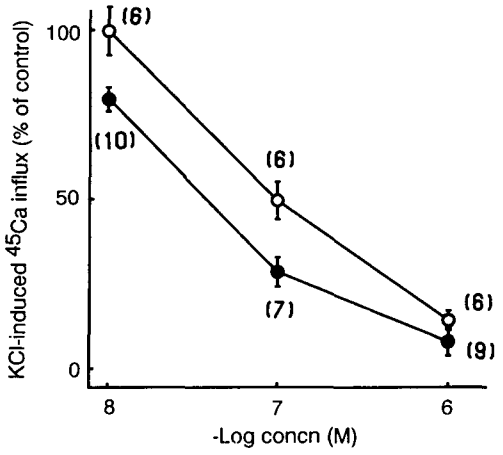

FIG. 5. Effect of KB-2796 on 50 mM KCl-induced ^{45}Ca influx in dog mesenteric (○) and cerebral (●) arteries. The residual ^{45}Ca influx in mesenteric and cerebral arteries were 161 ± 28 (n = 6) and 153 ± 13 (n = 10), respectively, in physiological salt solution; 307 ± 55 (n = 6) and 280 ± 18 nmol Ca^{2+} (g tissue)$^{-1}$ (n = 10), respectively, in 50 mM KCl solution. KCl-induced ^{45}Ca influx was calculated by subtracting the residual ^{45}Ca influx in normal solution from that in 50 mM KCl solution. KB-2796 was added 30 min before applying 50 mM KCl. The data are means ± s.e.m. Numbers beside the concentration-response curves indicate the number of preparations.

Discussion

The present results show that KB-2796 is a more potent inhibitor of both [^{3}H]NTD binding and KCl-induced contraction in dog vascular smooth muscle than either flunarizine or cinnarizine. KB-2796 also inhibits ^{45}Ca influx elicited by high KCl at the same concentration as that producing vasorelaxation. These observations demonstrate that KB-2796 is a potent L-type calcium channel antagonist in vascular smooth muscle.

High affinity binding sites for [^{3}H]NTD, which may be associated with voltage-dependent calcium channels, have been shown to exist in membrane preparations from several tissues (Glossmann & Ferry 1985; Vaghy et al 1987). In our study, [^{3}H]NTD was also found to bind to a single class of sites in dog aortic membranes, with a K_d of 0·41 nM and a B_{max} of 31 fmol (mg protein)$^{-1}$. These K_d and B_{max} values were nearly identical to those of a high affinity binding site reported by Triggle et al (1982) in dog aortic and mesenteric membranes. KB-2796 and other diphenylpiperazines inhibited [^{3}H]NTD binding to dog aortic membranes, apparently in the same manner as nicardipine. This result is comparable with our previous data obtained in guinea-pig cerebral cortex membranes (Iwamoto et al 1988b). In that study, we suggested that KB-2796 might inhibit the 1,4-dihydropyridine binding through a negative allosteric mechanism, since KB-2796 increased the rate of dissociation of [^{3}H]NTD from the binding site.

As noted above, there is a correlation between the potency of 1,4-dihydropyridines as vasorelaxants and their ability to inhibit [^{3}H]NTD binding (Bolger et al 1983; Janis et al 1984; Goll et al 1986). Similar investigations have also been done with phenylalkylamine derivatives (Goll et al 1986; Zobrist et al 1986) and benzothiazepine derivatives (Schoemaker et al 1987). We examined whether the interaction of KB-2796 and other diphenylpiperazines with calcium channels leads to vasorelaxation. In the present study, dog isolated mesenteric arteries, which have been shown to have binding properties similar to the aorta (Triggle et al 1982), were used to measure vasorelaxation. In dog arterial smooth muscle, KCl-induced contraction is particularly sensitive to voltage-dependent calcium entry and is easily inhibited by calcium channel antagonists (Shimizu et al 1980; Yamamoto et al 1983). As summarized in Fig. 4, a good correlation was obtained between inhibitory potencies for [^{3}H]NTD binding and high KCl-induced contraction among the 6 compounds of diphenylpiperazine derivatives. Thus, according to the present results the inhibition of [^{3}H]NTD binding by these derivatives is probably responsible for their vasorelaxing activity, and KB-2796 is one of the most potent inhibitors among them.

Calcium antagonists such as nifedipine (Godfraind 1983) and flunarizine (Godfraind & Dieu 1981) have been found to inhibit ^{45}Ca influx induced by KCl. KB-2796 also inhibited KCl-induced ^{45}Ca influx in both dog mesenteric and cerebral arteries, indicating that KB-2796 inhibits calcium entry through voltage-dependent calcium channels. Furthermore, in mesenteric arteries, the concentration of KB-2796 required to inhibit high KCl-induced ^{45}Ca influx was similar to the concentration required to inhibit KCl-induced contraction. In contrast, KB-2796 did not inhibit ^{45}Ca influx through passive leak channels in resting arteries. These findings suggest that KB-2796 is a specific voltage-dependent calcium antagonist in vascular smooth muscle.

KB-2796 has been reported to increase selectively vertebral blood flow in anaesthetized dogs (Kanazawa et al 1990) and to inhibit K^+- and prostaglandin $F_{2\alpha}$-induced contraction of cerebral arteries more strongly than those of peripheral arteries (Kanazawa & Toda 1987). In this study, KB-2796 was also found to inhibit KCl-induced ^{45}Ca influx in cerebral arteries more potently than in mesenteric arteries.

It has been established (Triggle & Janis 1984; Miller & Freedman 1984) that [^{3}H]NTD binds in a similar way to smooth muscle, cardiac muscle and neuronal tissue of several species. Diphenylpiperidine derivatives such as fluspirilene and pimozide, possessing a calcium antagonistic action (Gould et al 1983; King et al 1989), were also found to inhibit

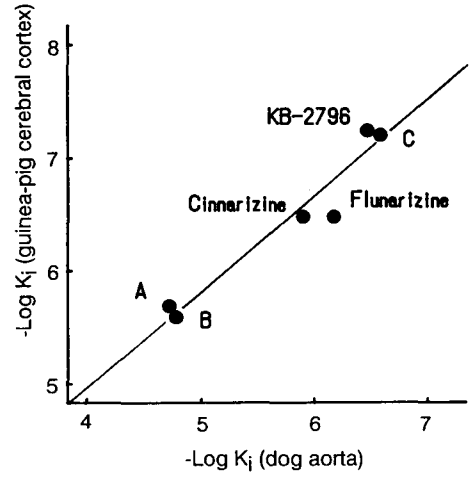

FIG. 6. Relationship between the inhibition (K_i) of [^{3}H]NTD binding in dog aorta and in guinea-pig cerebral cortex (Iwamoto et al 1988b). The correlation coefficient (r) is 0·97 ($P < 0·01$).

[^{3}H]NTD binding more strongly in cerebral cortex membranes than in cardiac membranes (Quirion et al 1985). Therefore, using the present results in combination with our previously published data (Iwamoto et al 1988b), we compared the inhibition of [^{3}H]NTD binding in the dog aorta to that in the guinea-pig cerebral cortex, and found a strong correlation (Fig. 6) for the six diphenylpiperazine compounds. However, K_i values of these compounds obtained in the cerebral cortex were 4–9 times greater than those in the aorta. This indicates that the binding affinities of KB-2796 and other diphenylpiperazines are greater in the cerebral cortex than in the aorta. In contrast, nicardipine had similar affinities in the aorta and cerebral cortex. Thus, KB-2796 and diphenylpiperazines, in contrast to 1,4-dihydropyridines, may act preferentially on calcium channels in the cerebral cortex. The relative selectivity of the diphenyl-piperazines for neuronal tissue might be related to the effectiveness of KB-2796 in treating neuronal impairment following ischaemia (Harada et al 1988, 1989; Yoshidomi et al 1989).

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