Neurochemical Investigation on the Effects of a New Diphenylpiperazine Calcium Antagonist, KB-2796, on the Central Dopaminergic System of Rats

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ABSTRACT — The effects of KB-2796, a new diphenylpiperazine calcium antagonist, on the striatal dopaminergic system of rats were investigated in comparison with various calcium antagonists and the dopamine antagonist chlorpromazine. The inhibiting effect of KB-2796 on [3H]spiperone binding to striatal membranes in vitro was weaker than those of chlorpromazine and the other diphenylpiperazine analogues, flunarizine and cinnarizine, and more potent than those of verapamil and nicardipine. Diltiazem and nifedipine were inactive. KB-2796 (30, 100 mg/kg, p.o.) had no effect on Kd and Bmax values of in vitro [3H]spiperone specific binding to striatal membranes obtained from the rat at 36 hr and 7 days after repeated administration for 18 days, whereas flunarizine (30 mg/kg, p.o.) and chlorpromazine (3 mg/kg, p.o.) increased Bmax values by 47% and 31%, respectively, at 36 hr, but not at 7 days after the final administration. At 1 hr after the single administration, KB-2796 (30, 100 mg/kg, p.o.) had no effect on the content of dopamine and its metabolites in the striatum, whereas flunarizine (30 mg/kg, p.o.) and chlorpromazine (3 mg/kg, p.o.) increased the level of homovanillic acid. These results indicate that flunarizine may affect dopaminergic neurotransmission by partially blocking dopamine D2 receptors, while KB-2796 has negligible in vivo effect on the dopaminergic system.

Calcium antagonists are drugs widely prescribed for cardiovascular and cerebrovascular disorders. Their therapeutic effectiveness relies on their ability to block calcium entry through voltage sensitive calcium channels. Recently, side effects such as extrapyramidal symptoms and depression have been observed in patients, especially in elderly patients, treated with calcium antagonists such as flunarizine and cinnarizine (1, 2). Neuroleptics such as chlorpromazine are known to elicit extrapyramidal side effects by blocking postsynaptic dopamine D2 receptors in the striatum (3, 4). Therefore, interaction of these calcium antagonists with the dopaminergic transmission system is thought to be a possible mechanism of occurrence of the side effects.

In recent years, several investigators have demonstrated that some calcium antagonists inhibit dopamine release (5, 6) and directly interact with dopamine recognition sites, thereby possibly affecting dopaminergic transmission (7). Flunarizine, a diphenylpiperazine calcium antagonist, inhibits dopamine release from rat striatal slices in vitro (8) and [3H]spiperone binding to rat striatal homoge-
nate (9). Govoni et al. (10) have reported that flunarizine and nimodipine, a dihydropyridine calcium antagonist, increase the numbers of $[^3H]$spiroperidol binding sites with a reduction of the binding affinity after repeated administration in rats. Furthermore, the binding affinity of flunarizine for dopamine D$_2$ receptors has been shown to be higher than that for the Ca$^{2+}$ channel sites (11).

KB-2796, 1-[bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl)piperazine dihydrochloride, is a new diphenylpiperazine calcium antagonist which exhibits selective vasodilator activity on cerebral vessels (12). KB-2796 displaces $[^3H]$nitrendipine binding to homogenates of guinea pig cortex (13) and canine vessels (14). We have previously reported that KB-2796 does not inhibit apomorphine-induced stereotyped behaviors such as climbing and circling in mice, but it inhibits methamphetamine-induced locomotion and circling at a high dose (15). However, the neurochemical mechanism of KB-2796 on the central dopaminergic system is not well known. There are only a few reports on the effect of the in vivo chronic treatment with calcium antagonists on dopaminergic receptors (10). In this study, therefore, we examined the binding profile of KB-2796 to dopamine D$_2$ receptors and its effect on dopamine metabolism in the striatum of rats to determine its possible influence on dopaminergic transmission.

MATERIALS AND METHODS

Animals
Male Wistar rats (Japan SLC, Inc.) weighing 200–220 g were used. Animals were housed in a temperature- and humidity-controlled room (12 hr light and 12 hr dark cycle) with free access to food and water.

Drugs
KB-2796 was synthesized and verapamil and diltiazem were extracted from Wasoran tablets (Eisai Co. Ltd.) and Helvessor tablets (Tanabe Co. Ltd.), respectively, by the New Drug Research Laboratories of Kanebo. The following drugs were purchased from Sigma: flunarizine dihydrochloride, cinnarizine, nifedipine, nicardipine hydrochloride, chlorpromazine hydrochloride, pargyline hydrochloride and caffic acid. (+)-[Phenyl-4-$^3$H]spiperone (specific activity, 1.07 TBq/mmol) was obtained from Amersham; (+)-butaclamol, from Research Biochemicals; and sodium octyl sulfate, from Nacalai Tesque.

Preparation of membrane and binding assay
Preparation of membrane and $[^3H]$spiperone binding assays were performed according to the method reported by Govoni et al. (10) with minor modifications. Rats were decapitated and their brains were quickly removed. Striatal tissues were dissected and frozen in liquid nitrogen and kept at −80°C until assayed. Tissues were homogenized in 30 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4), washed by centrifugation at 30,000 × g, and finally resuspended in 40 volumes of 50 mM Tris-HCl buffer, pH 7.4 (buffer B) containing 120 mM NaCl, 5 mM HCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 0.1% ascorbic acid and 10 μM pargyline.

For determining the in vitro effects of drugs on dopamine D$_2$ receptors, binding assays were performed by incubating 1.9 ml of buffer B containing $[^3H]$spiperone (final concentration: 0.4 nM) and each drug at various concentrations with 0.1 ml of crude synaptic membranes (final concentration: 1.25 mg-tissue/ml) at 37°C for 20 min. (+)-Butaclamol (10 μM) was used to define non-specific binding. The binding assay was terminated by rapid vacuum filtration through Whatman GF/C glass fiber filters.

For determining the in vivo chronic effects of drugs on dopamine D$_2$ receptors, drugs suspended in 5% arabic gum were orally administered to a group of 5 rats once a day for 18 days. The animals were killed 36 hr and 7 days after the final administration of drugs, according to the method reported by Govoni et al. (10). Striatal tissues and membrane fractions were obtained, and binding assays were per-
formed by the same method described above, except that 6 concentrations of [³H]spiperone ranging from 0.0125 to 0.7 nM were used. The dissociation constant (Kd) and the density of binding sites (Bmax) were determined by Scatchard analysis (16). Protein content was determined according to Lowry et al. (17).

**Determination of the content of dopamine and its metabolites**

A group of 5 fasted rats were orally administered drugs suspended in 5% arabic gum. One hour after administration, the animals were killed by decapitation. Their brains were removed, and the striatal tissues were dissected on ice, frozen in liquid nitrogen, and stored at -80°C until assayed.

The striatal content of dopamine and its metabolites was measured by the method of Warnhoff (18). The tissues were homogenized in 10 volumes of 0.05 M perchloric acid containing 0.0032% EDTA-2Na and 1/10 volumes of caffeic acid (3 μM) as an internal standard, and the homogenate was centrifuged at 20,000 × g at 4°C for 15 min. High pressure liquid chromatography (HPLC) was performed on 20 μl of supernatant, using an electro-chemical detector (ECD).

**RESULTS**

**In vitro effects of calcium antagonists on [³H]spiperone binding**

As shown in Fig. 1, among the calcium antagonists tested, KB-2796, flunarizine, cinnarizine, verapamil and nicardipine inhibited [³H]spiperone binding to striatal membranes in a concentration-dependent manner. Diltiazem also slightly inhibited binding; however, the Kᵢ value was greater than 15,000 nM. Nifedipine had almost no effect on displacement of binding, with inhibition of 7% at 10⁻⁴ M.

The Kᵢ values and Hill coefficients for each drug are shown in Table 1. Flunarizine, the most potent drug among the calcium antagonists tested, was approximately 5 times less potent in inhibiting [³H]spiperone binding, and KB-2796 was approximately 35 times less potent than chlorpromazine. The Hill coefficient for KB-2796, flunarizine, cinnarizine and verapamil were close to 1, whereas the coefficient for nicardipine was less than 1.

**Effects of repeated administration of KB-2796, flunarizine and chlorpromazine on [³H]spiperone binding**

Figures 2 and 3 show representative data of
Scatchard analysis on[^3]H]spiperone binding to rat striatal membranes obtained 36 hr and 7 days, respectively, after repeated administration of KB-2796, flunarizine and chlorpromazine for 18 days. Tables 2 and 3 represent the average K_d and B_max values obtained from 4 experiments. KB-2796 at 30 and 100 mg/kg did not affect K_d and B_max values. Flunarizine at 30 mg/kg and chlorpromazine at 3 mg/kg significantly increased B_max values by 47% (P < 0.01) and 31% (P < 0.01), respectively, after 36 hr of washing out the drugs, whereas K_d values were not changed by administration. After 7 days of washing out the drugs, all changes in the binding parameters had disappeared.

**Table 1. Inhibition by various drugs of[^3]H]spiperone (0.4 nM) specific binding to rat striatal membranes**

| Drugs         | K_i values (nM) | Hill coefficients |
|---------------|-----------------|-------------------|
| Chlorpromazine| 3.83 ± 0.22     | 0.97 ± 0.04       |
| Flunarizine   | 15.7 ± 1.56     | 1.04 ± 0.08       |
| Cinnarizine   | 79.1 ± 3.97     | 0.96 ± 0.12       |
| KB-2796       | 116 ± 4.34      | 1.29 ± 0.07       |
| Verapamil     | 1,053 ± 218     | 1.11 ± 0.07       |
| Nicardipine   | 3,428 ± 978     | 0.70 ± 0.14       |
| Diltiazem     | > 15,000        |                   |
| Nifedipine    | > 15,000        |                   |

Each value represents the mean ± S.E. of 3 experiments performed with duplicate samples.

**Fig. 2.** Effect of KB-2796 (--- ▲ --- 30 mg/kg, --- △ --- 100 mg/kg), flunarizine (--- ■ --- 30 mg/kg) and chlorpromazine (--- □ --- 3 mg/kg on[^3]H]spiperone specific binding at 36 hr after repeated administration for 18 days. Each point is the mean of duplicate determinations in a representative experiment. The lines determined by linear regression analysis of Scatchard plots are indicated. --- ○ --- control.

**Fig. 3.** Effect of KB-2796, flunarizine and chlorpromazine on[^3]H]spiperone binding at 7 days after repeated administration for 18 days. For further explanations, see Fig. 2.
Effects of single administration of KB-2796, flunarizine and chlorpromazine on the content of dopamine and its metabolites

Table 4 shows the content of dopamine and its metabolites in rat striatum 1 hr after a single administration of KB-2796, flunarizine or chlorpromazine. KB-2796 did not significantly affect the content of dopamine, dihydroxyphenyl acetic acid (DOPAC), homovalinic acid (HVA) and 3-methoxytyramine (3-MT), even at a dose of 100 mg/kg. Flunarizine at 30 mg/kg and chlorpromazine at 3 mg/kg signifi-
cantly increased HVA content by 26% (P < 0.05) and 24% (P < 0.05), respectively.

DISCUSSION

KB-2796 displaces [3H]spiperone binding to dopamine D2 receptors with an intermediate potency among the calcium antagonists tested. The potency of KB-2796 was weakest among the other diphenylpiperazine analogues such as flunarizine and cinnarizine. Furthermore, KB-2796 influenced neither the binding profile of [3H]spiperone after repeated administration for 18 days nor dopamine metabolism even at a high dose of 100 mg/kg, p.o. We have reported that KB-2796 and flunarizine protect against KCN-induced death in mice, with ED50 values of 40 and 28 mg/kg, p.o. (19). Furthermore, we have reported that KB-2796 at 100 mg/kg, p.o. does not affect apomorphine-induced stereotyped behavior such as cage climbing and turning and the content of dopamine and its metabolites and dopamine turnover rate in mice (15). Flunarizine at 30 mg/kg, p.o. inhibits these behavioral changes and increase the content of the dopaminergic system (15). The LD50 value for KB-2796 is 506 mg/kg, p.o. in male rats (T. Unno et al., unpublished data). These results may indicate that although KB-2796 indeed has weak binding affinity to dopamine D2 receptors in vitro, its interaction with dopamine D2 receptors is negligible after systemic administration in vivo.

However, it remains to be determined why KB-2796 did not affect in vivo dopaminergic neurotransmission in spite of exhibiting a weak activity in displacing [3H]spiperone binding to dopamine D2 receptors. Pharmacokinetic parameters of KB-2796 such as absorption, penetration into the brain, and metabolism may be possible factors which influence the in vivo action of the drug. Waki et al. (20) have reported that KB-2796 and flunarizine administered orally penetrate into the brain of rats. In fact, KB-2796 as well as nicardipine, which is the weakest displacer of [3H]spiperone binding in the present experiment, inhibit metham-

**Table 2. Effect of KB-2796, flunarizine and chlorpromazine on [3H]spiperone binding at 36 hr after repeated administration for 18 days**

| Drug        | Dose (mg/kg, p.o.) | Kd (nM) | Bmax (fmol/mg-protein) |
|-------------|-------------------|---------|-----------------------|
| Control     |                   | 0.073 ± 0.011 | 441 ± 23             |
| KB-2796     | 30                | 0.100 ± 0.011 | 469 ± 9              |
|             | 100               | 0.075 ± 0.006 | 494 ± 10             |
| Flunarizine | 30                | 0.081 ± 0.006 | 649 ± 33**           |
| Chlorpromazine | 3               | 0.085 ± 0.004 | 576 ± 22**           |

Each value represents the mean ± S.E. (n = 4). **: P < 0.01 vs. control (Dunnett’s multiple range test).

**Table 3. Effect of KB-2796, flunarizine and chlorpromazine on [3H]spiperone binding at 7 days after repeated administration for 18 days**

| Drug        | Dose (mg/kg, p.o.) | Kd (nM) | Bmax (fmol/mg-protein) |
|-------------|-------------------|---------|-----------------------|
| Control     |                   | 0.097 ± 0.009 | 447 ± 15             |
| KB-2796     | 30                | 0.086 ± 0.013 | 464 ± 14             |
|             | 100               | 0.097 ± 0.010 | 440 ± 10             |
| Flunarizine | 30                | 0.094 ± 0.009 | 454 ± 13             |
| Chlorpromazine | 3               | 0.090 ± 0.014 | 475 ± 24             |

Each value represents the mean ± S.E. (n = 4).
amphetamine-induced locomotion and circling in mice (17). This kind of inhibitory effect has also been reported with other calcium antagonists such as flunarizine and nifedipine (21) and verapamil (22). In amphetamine-induced circling, calcium is supposed to play an important role (22). These facts may indicate that KB-2796 given orally can penetrate into the brain. However, the concentration in the brain after oral administration of KB-2796 could not reach a sufficient level to exert its effects on dopamine D2 receptors.

Of the calcium antagonists tested in the present study, flunarizine was found to be one of the most potent displacers of [3H]spiperone binding to dopamine D2 receptors in vitro and found to increase B$_{\text{max}}$ values after repeated administration without changing K$_d$ values as observed with chlorpromazine. On the other hand, Govoni et al. (10) also observed an increase in the B$_{\text{max}}$ and K$_d$ values with repeated administration of flunarizine. This discrepancy in the K$_d$ value cannot be explained at present. The K$_d$ value may be possibly influenced by the amount of drug remaining in the brain after some washout periods. Moreover, a difference in the strain of rats used may be a possible cause of the discrepancy.

Govoni et al. (10) supposed that normal nerve function might be modified by in vivo chronic blockade of calcium antagonist-sensitive, voltage-dependent calcium channels, since nimodipine which has no binding affinity to dopamine D$_2$ receptors increased K$_d$ and B$_{\text{max}}$ values for [3H]spiperone binding. In the present experiment, KB-2796 did not affect the binding characteristics of [3H]spiperone after chronic administration. KB-2796 is about three times more potent than flunarizine in displacing [3H]nitrendipine binding to guinea pig cortex membrane (13). Although we did not measure the brain concentration of KB-2796 in the present experiments, as aforementioned, KB-2796 can penetrate into rat brain and is expected to exert its calcium antagonistic activity in vivo. Therefore, the change in dopaminergic transmission by in vivo chronic administration of calcium antagonists cannot be simply explained by the blockade or the modification of calcium antagonist-sensitive, voltage-dependent calcium channels. On this point, we need further studies to determine the role of calcium channels in the modulation of dopaminergic neurotransmission.

These results indicated that KB-2796 may have negligible influence on in vivo central dopaminergic neurotransmission and may be expected to cause less extrapyramidal side effects than flunarizine.

| Drugs      | Dose (mg/kg, p.o.) | DA   | DOPAC | HVA  | 3-MT  |
|------------|-------------------|------|-------|------|-------|
| Control    | —                 | 60.0 ± 4.3 | 16.3 ± 0.9 | 5.37 ± 0.22 | 2.47 ± 0.17 |
| KB-2796    | 30                | 62.3 ± 5.2 | 18.4 ± 1.0 | 5.47 ± 0.24 | 2.32 ± 0.17 |
|            | 100               | 54.6 ± 6.1 | 15.6 ± 1.6 | 5.13 ± 0.30 | 1.87 ± 0.22 |
| Flunarizine| 30                | 70.4 ± 7.5 | 20.4 ± 0.9 | 6.78 ± 0.35* | 2.56 ± 0.24 |
| Chlorpromazine | 3              | 64.8 ± 4.9 | 18.7 ± 1.2 | 6.66 ± 0.44* | 2.27 ± 0.13 |

Each value represents the mean ± S.E. (n = 5). *: P < 0.05 vs. control (Dunnett's multiple range test). DA: dopamine, DOPAC: 3,4-dihydroxyphenyl-acetic acid, HVA: homovanillic acid, 3-MT: 3-methoxytyramine.
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