Effect of 5-{3-[4-(4-Fluorophenyl)-1-Piperazinyl]-Propoxy}indan (BP-528) on Benzodiazepine Receptor Bindings and γ -Aminobutyric Acid Release

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Abstract—The action of a new type of anti-anxiety compound, 5-{3-[4-(4-fluorophenyl)-1-piperazinyl]-propoxy}indan (BP-528), was tested on benzodiazepine receptor bindings and on [3 H]-GABA release. BP-528 did not alter [3 H]-diazepam binding to rat cerebral cortical and hippocampal membranes either in the presence or absence of GABA; and the binding of [3 H]-propyl- β -carboline-3-carboxylate at low concentration (0.04 nM), which labels only the type I benzodiazepine receptor, was not changed by BP-528. BP-528 did not interact with the GABA-benzodiazepine receptor complex, which is related to the anti-anxiety activity of benzodiazepines. This compound affected neither GABA binding nor GABA uptake. Ten micromolar BP-528 depressed high K^+ -induced [3 H]-GABA release from preloaded rat hippocampal slices. However, the same concentration of BP-528 also inhibited high K^+ -induced calcium uptake by rat cerebral cortical synaptosomes.

The indan derivative 5-{3-[4-(4-fluorophenyl)-1-piperazinyl]-propoxy}indan (BP-528) has been shown to have anti-anxiety properties with weak muscle-relaxant effects in an animal test system (1). The mechanism by which BP-528 produces the anti-anxiety effect has yet to be elucidated.

Benzodiazepines, the widely-used and well-investigated drugs with anxiolytic, anti-convulsive, and muscle-relaxant effects, are reported to bind to high affinity receptor sites in the central nervous system with a chemical specificity that correlates well with the anxiolytic activity (2, 3). Certain compounds possessing anxiolytic activity, which are chemically unrelated to benzodiazepines, bind to benzodiazepine receptors (4–8). The benzodiazepine receptors are thought to be partly coupled with GABA-benzodiazepine receptor binding sites and to modulate the transmission by GABA at the postsynaptic level (9). In addition, diazepam, a representative benzodiazepine, caused a significant dose-dependent inhibition of the elevated K^+ -evoked release of [3 H]-GABA by acting

at the presynaptic level (10). These findings show that the GABA-ergic system in the central nervous system relates to the pharmacological effects of benzodiazepines. In this study, we examined the effect of BP-528 on the GABA-ergic system, benzodiazepine receptor bindings and GABA release.

Male Sprague-Dawley rats (weighing 150–250 g) were decapitated, and their brains were rapidly removed and dissected. The cerebral cortex and hippocampus were immediately homogenized in 100 vol. of ice-cold 50 mM Tris-citrate buffer (pH 7.4) for [3 H]-diazepam binding and 50 mM Na/K phosphate buffer (pH 7.4) for [3 H]-propyl- β -carboline-3-carboxylate ([3 H]-PCC) binding. The homogenate was centrifuged at 20,000 g for 15 min. The pellet was washed twice and suspended in 100 vol. of the homogenizing buffer. The suspension was frozen, thawed and washed. This freezing-thawing-washing cycle was repeated three times to remove endogenous GABA (11). The final pellet was suspended in the same buffer and used for binding assay. Specific

bindings of [^3H]-diazepam and [^3H]-PCC to membranes were determined as described elsewhere (12).

Release of [^3H]-GABA from the preloaded slices of rat hippocampus was performed by the superfusion method as described before (13). The slices (0.25 mm thick) of hippocampus were prepared by a mechanical tissue chopper. The slices were suspended in Krebs-Ringer HEPES buffer (KR), containing 138 mM NaCl, 5.6 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 11 mM NaHCO_3 , 1 mM NaH_2PO_4 , 20 mM HEPES buffer (pH 7.4). The slices in KR were preincubated for 20 min at 37°C. Then the medium was replaced with fresh KR containing 10 μM [^3H]-GABA (8 μCi) and 0.1 mM aminooxyacetic acid, and the suspension was incubated for another 10 min. Superfusion of the loaded slices were done with KR at a constant flow rate of 2.0 ml/min in separate chambers (volume, 0.6 ml) at 37°C with gentle shaking. Release was induced by flowing high K^+ -KR (25 mM K^+ , prepared by equimolar replace-

ment of NaCl) for 2 min, twice, with a 8 min interval. The release of radioactivity in excess of basal efflux resulting from stimulation with 25 mM K^+ was calculated as the percentage of the total release activity taken up by the slices. Values for the release activity represents the ratio of the percentage of radioactivity released during the first and second stimulation (S_2/S_1).

Preparation of synaptosomes and $^{45}\text{Ca}^{2+}$ uptake by the synaptosomes were carried out as described elsewhere (14). Protein was measured by the method of Lowry et al. (15).

[^3H]-Diazepam (76.7 Ci/mmol), [^3H]-PCC (9.51 Ci/mmol), and $^{45}\text{CaCl}_2$ were purchased from New England Nuclear. [^3H]-GABA (65.3 Ci/mmol) was from Amersham International. BP-528 was a generous gift from Mitsubishi Chemical Industries Co., Ltd., through Dr. Akihiro Tobe. All other reagents were obtained from commercial sources.

We examined the effects of BP-528 on [^3H]-diazepam and [^3H]-PCC bindings to

Table 1. Effect of BP-528 on [^3H]-diazepam and [^3H]-propyl- β -carboline-3-carboxylate ([^3H]-PCC) bindings to membranes of rat cerebral cortex and hippocampus

	Specifically bound [^3H]-diazepam (fmole/mg protein)		
	None	10 μM GABA	
Cortex			
Control	193.4 $\pm$ 11.8	375.1 $\pm$ 23.3	(5)
BP-528 (10 μM)	198.2 $\pm$ 12.8	342.5 $\pm$ 30.2	(5)
Hippocampus			
Control	171.8 $\pm$ 12.8	245.3 $\pm$ 16.1	(13)
BP-528 (10 μM)	168.3 $\pm$ 13.0	238.6 $\pm$ 16.6	(12)

	Specifically bound [^3H]-PCC (fmole/mg protein)		
	None	10 μM GABA	
Cortex			
Control	61.6 $\pm$ 6.5	71.2 $\pm$ 8.1	(6)
BP-528 (10 μM)	57.2 $\pm$ 5.5	65.9 $\pm$ 7.1	(6)
Hippocampus			
Control	63.3 $\pm$ 8.3	69.5 $\pm$ 8.5	(4)
BP-528 (10 μM)	54.7 $\pm$ 4.1	62.2 $\pm$ 6.5	(4)

Cerebral cortex and hippocampus were homogenized in the homogenizing buffer, and the membranes were prepared as described in the text. Binding experiments were performed by incubating 2 nM [^3H]-GABA or 0.04 nM [^3H]-PCC and aliquots of membranes with or without 10 μM GABA for 30 min at 4°C. Results are the means $\pm$ S.E.M. of the number of experiments shown in parenthesis.

rat cerebral cortical and hippocampal membranes (Table 1). Consistent with previous data (9, 16–18), binding of [^3H]-diazepam was enhanced by 10 μM GABA, but that of [^3H]-PCC was not. BP-528 at 10 μM had no effect on [^3H]-diazepam and [^3H]-PCC bindings to either type of membrane in the presence or absence of GABA. We also examined the effect of BP-528 from 1 μM to 30 μM , but it had no effect on either of the bindings (data not shown). Therefore, we next investigated the effect of BP-528 on binding, uptake, and release of GABA in the central nervous system. BP-528 (10 μM) did not change GABA binding to rat cerebral cortical membranes and GABA uptake by rat synaptosomes (data not shown). Table 2A shows the effect of BP-528 on [^3H]-GABA release induced by high K^+ medium from the preloaded rat hippocampal slices. Therefore, the ratio of [^3H]-GABA release at the second high K^+ -stimulation to that at the first one (S_2/S_1) corresponds with that in our previous study (13). Addition of 10 μM BP-528 at the second stimulation significantly reduced the release of [^3H]-GABA compared with the control.

Release of [^3H]-GABA induced by high K^+ medium is dependent on extracellular calcium ion and calcium influx through

opened voltage-sensitive calcium channels (19–22). To determine whether the inhibitory effect of BP-528 is due to its action on calcium influx, we examined the effect of BP-528 on high K^+ -induced calcium uptake by rat cerebral cortical synaptosomes. BP-528 inhibited high K^+ -induced calcium uptake by synaptosomes in a dose-dependent manner (Table 2B). BP-528 did not affect the calcium uptake by non-depolarized synaptosomes at concentrations up to 30 μM (data not shown).

In the present study, we sought to determine whether antianxiety activity of BP-528 could be explained by its action on benzodiazepine receptors in the brain membranes. Two [^3H]-ligands, [^3H]-diazepam and [^3H]-PCC, were used because receptor subtypes were distinguishable using them. Benzodiazepine receptor subtypes have been classified as type I and II (18). [^3H]-Diazepam is thought to bind to both receptor subtypes, and [^3H]-PCC at low concentrations is thought to only bind to type I receptor. Furthermore, the anti-anxiety activity of benzodiazepines is thought to be mediated through type I receptor (18, 23). BP-528 at 10 μM did not change bindings of [^3H]-diazepam or bindings of [^3H]-PCC to both cerebral cortical and hippocampal mem-

Table 2. Effects of BP-528 on high K^+ -induced [^3H]-GABA release from the preloaded rat hippocampal slices and high K^+ -induced calcium uptake by rat cerebral cortical synaptosomes

A) _____ High K^+ -induced [^3H]-GABA release (S_2/S_1) _____	
Control	0.621 $\pm$ 0.023
BP-528 (10 μM)	0.415 $\pm$ 0.040*
B) _____ High K^+ -induced calcium uptake (nmol/mg protein) _____	
Control	3.68 $\pm$ 0.12
BP-528 (3 μM)	3.31 $\pm$ 0.22
(10 μM)	2.94 $\pm$ 0.11*
(30 μM)	2.68 $\pm$ 0.11**

A) [^3H]-GABA release from the preloaded hippocampal slices (0.25 mm) was examined in the presence of 0.1 mM aminooxyacetic acid by the superfusion method as described elsewhere (13). Release was induced by flowing 25 mM K^+ (for 2 min), two times, with 8 min interval. During the second stimulation period, BP-528 (10 μM) was present in the superfusion fluid. Values for [^3H]-GABA release were represented by the ratio of the radioactivity release at the second stimulation to that at the first one (S_2/S_1). Values are means $\pm$ S.E.M. of 4 experiments. B) Calcium uptake by synaptosomes was examined as described before (14). High K^+ -induced calcium uptake was defined as the difference between calcium present in the control (5.6 mM K^+) and the depolarized (56 mM K^+) synaptosomes. Values are means $\pm$ S.E.M. of 4 experiments. * P <0.01, ** P <0.001, compared with the control.

branes. This compound also failed to alter the substantial stimulation of [^3H]-diazepam binding by GABA to either type of membrane. Moreover, [^3H]-GABA binding was not affected by BP-528. These findings suggest that BP-528 does not interact with either type of benzodiazepine receptor or GABA receptor in the central nervous system.

We demonstrated that BP-528 reduced high K^+ -induced [^3H]-GABA release from the preloaded rat hippocampal slices and high K^+ -induced calcium uptake by rat synaptosomes. It is accepted that high K^+ -induced influx of calcium is essential for GABA release. The inhibition of GABA release might be caused mainly by the reduction by BP-528 of calcium influx into nerve endings. The findings that BP-528 affected only high K^+ -induced calcium uptake but not that under the non-depolarizing condition indicate that BP-528 selectively inhibits the voltage-sensitive calcium channel. Because many groups of drugs, analgesics, anticonvulsives, antidepressants, neuroleptics, etc., are known to inhibit depolarization induced calcium uptake (14, 24–28), it is not known whether the inhibitory effects of BP-528 on calcium influx are related to its anti-anxiety properties.

The results in this study shows that BP-528 did not interact with the GABA-benzodiazepine receptor system, which is related to the anti-anxiety activity of benzodiazepines. The mechanism for the anti-anxiety properties of BP-528 may be different from those of benzodiazepines.

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