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PROTECTION WITH PHENCYCLIDINE AGAINST INACTIVATION OF 5-HT$_2$ RECEPTORS BY SULFHYDRYL-MODIFYING REAGENTS

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Abstract—We investigated whether phencyclidine (PCP)-induced head-twitch was antagonized in rats by ritanserin, a selective serotonin$_2$ (5-HT$_2$) receptor antagonist, to confirm the involvement of 5-HT neurons in PCP action and to discover whether PCP could protect the binding sites of [^{3}H]PCP and [^{3}H]ketanserin from the inhibitory effect of protein-modifying reagents which affect sulfhydryl groups. PCP (7.5, 10 and 12.5 mg/kg, i.p.)-induced head-twitch was completely antagonized by ritanserin (1 mg/kg, s.c.). Scatchard plots of specific [^{3}H]PCP and [^{3}H]ketanserin binding showed that sulfhydryl-modifying reagent, *N*-ethylmaleimide (NEM, 100 μM) caused a significant decrease in B_{max} without changing K_d. PCP (10 μM) and ritanserin (1 μM) protected [^{3}H]PCP and [^{3}H]ketanserin binding sites from the decrease in the number induced by NEM (100 μM). 5-HT protected [^{3}H]5-HT binding sites from inactivation by NEM, but PCP and ritanserin did not show any effect. On the basis of the present findings, it is concluded that PCP can interact with 5-HT$_2$ receptors directly or allosterically, and 5-HT$_2$ receptors may locate at PCP binding sites in membranes.

Phencyclidine ("PCP" or "angel dust") is a psychotomimetic drug that is abused in epidemic proportions in some countries [1]. The schizophrenia-like syndrome that sometimes occurs with PCP abuse [2] or that is seen as a hallucinogenic reaction to general anesthesia [3] has stimulated a search for the mechanisms producing these adverse effects [4–5].

As Tricklebank [7] has reported, the "Serotonin syndrome" (head-weaving, forepaw treading and hind-limb abduction) develops when rats are given the proposed 5-HT$_{1A}$ selective agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), and this is blocked by pindolol [8, 9] and propranolol [10] which have been reported to have a high affinity for 5-HT$_{1A}$ sites, but not by ritanserin, a 5-HT$_2$-specific antagonist [10]. The behavioral syndrome can be also produced by direct 5-HT receptor agonists such as 5-methoxy-*N*,*N*-dimethyltryptamine (5-MeODMT) or by drugs such as *p*-chloroamphetamine that release 5-HT from the presynaptic neurons. This behavioral syndrome is similar to some components of PCP-induced stereotypes [11]. We have suggested that PCP-induced stereotyped behavior including head-weaving, turning, backpedalling are mediated by serotonergic neurons since lesioning of the striatum with a specific serotonergic neuronal toxin (5,6-dihydroxytryptamine) or electrolytic lesioning of the raphe nucleus, which contains 5-HT cell bodies, diminishes PCP-induced stereotyped behavior [12, 13]. We have also suggested that PCP interacts with the 5-HT$_2$ receptor since it inhibits [^{3}H]spiperone binding to the 5-HT$_2$ receptor [14, 15] *in vitro* and decreases the number of 5-HT$_2$ receptors when given chronically *in vivo* [16].

Head-twitch can be produced in rats by the administration of hallucinogens such as LSD-25, mescaline and 2,5-dimethoxy-4-methoxyamphetamine [17] and in mice by PCP [18]. Hallucinogen-induced head-twitch is inhibited by methysergide and tends to be increased in intensity by *p*-chlorophenylalanine (PCPA) but is increased in intensity by median raphe lesions [17, 19, 20]. The increased incidence of head-twitch evoked by raphe lesions or PCPA treatment may be attributed to 5-HT receptor supersensitivity. Furthermore, the 5-HT antagonists such as metergoline, ketanserin, pipamperone and methysergide block the head-twitch response produced by 5-hydroxy-L-tryptophan and a 5-HT agonist, quipazine [21]. Drug affinities for 5-HT$_1$ receptors labeled by [^{3}H]5-HT could not be correlated with head-twitch blocking action [22]. In contrast, the ability of drugs to block head-twitch closely correlates with the affinity for 5-HT$_2$ receptors labeled by [^{3}H]spiperone [22].

In the present study, in order to gain an insight into the role of the 5-HT$_2$ receptor in the pharmacological action of PCP, we investigated whether PCP-induced head-twitch response was antagonized by ritanserin, a selective 5-HT$_2$ receptor antagonist [23], and whether PCP could protect the binding sites of [^{3}H]PCP and [^{3}H]ketanserin from an inhibitory effect of protein-modifying reagents which affect sulfhydryl groups.

METHODS

Animals. Male Fischer 344 rats (Charles River Breeding Co., Japan), weighing 150–200 g were used. The animals were maintained in a room with

controlled temperature, humidity (22–24°, 55 ± 5%), and light on 8.00–20.00 hr.

Drugs. Phencyclidine-HCl (PCP, synthesized by us, identified by NMR and IR) was dissolved in 0.9% saline. Ritanserin (Janssen–Kyowa) was dissolved in distilled water with a small amount of lactic acid, and sodium bicarbonate was used to bring the pH of the solution up to about 4. Other drugs were as follows: pargyline–HCl (Sigma, St Louis, MO), 5-hydroxytryptamine creatinine sulfate (5-HT, Sigma), methysergide hydrogen maleate (Sandoz, Basel), prazosin–HCl (Tokyo Chemical Industry), *N*-allylnormetazocine–HCl (SKF 10,047, NIDA), *N*-ethylmaleimide (NEM, Sigma), iodoacetamide (IOAA, Sigma), [^{3}H] 5-HT (specific activity 23.4 Ci/mmol, New England Nuclear, Boston, MA), [^{3}H] ketanserin (specific activity 78.6 Ci/mmole, New England Nuclear), [^{3}H] PCP (specific activity 50.0 Ci/mmol, New England Nuclear). The volume for i.p. injection was 0.2 ml/100 g body weight.

Behavioral studies. All behavioral experiments took place in a quiet room, at a temperature of 22–24° between 10.00 and 15.00 hr. The animals were observed while they were in a plastic cage with dimensions of 30 × 35 × 17 cm. The animals were habituated to the plastic cage by being placed individually in it for 30 min before the experiment and were randomly assigned to the various drug treatment groups. The animals were observed by one of the authors who was blind to the drug treatments. The 5-HT-mediated behaviors were evaluated by the method of Nabeshima *et al.* [24] and Lee *et al.* [25] with some modifications.

Rats were pretreated with vehicle or ritanserin (1 mg/kg, s.c.) 1 hr before administration of PCP.

The rating scales were as follows: head-weaving (the number of times the animal made slow, side to side or lateral head-movement), head-twitch (the number of times the animal's head twitched rapidly to the side, as in the pinna reflex), turning (the number of times the animal circled to left or right over 360° within a relatively small area) and backpedalling (the number of times the animal moved backward). Head-weaving and head-twitch induced by PCP were scored for 3 min every 15 min up to 2 hr post-injection. Turning and backpedalling induced by PCP were scored continuously up to 1 or 2 hr post-injection.

Tissue preparation and binding assay. Membrane preparation and ligand binding assay were carried out as previously described [14, 15, 26]. The rat was killed by decapitation. The whole brain excluding cerebellum was rapidly removed and synaptic membrane was prepared from the pooled brains of each group. The pooled brains were homogenized with 10 vol. of 0.32 M sucrose in 50 mM Tris–HCl buffer (pH 7.4 at 4°) and centrifuged at 1000 *g* for 10 min. The supernatant fraction was collected and centrifuged at 20,000 *g* for 20 min to obtain a pellet containing the crude mitochondrial fraction (P_2 fraction). The P_2 fraction was subjected to osmotic shock by adding 10 ml of distilled, deionized water. The suspension was centrifuged at 12,000 *g* for 20 min. The carefully-decanted supernatant was layered over a discontinuous gradient consisting of 0.6 and 1.0 M sucrose in 5 mM Tris–HCl buffer (pH 7.4 at 4°), and centrifuged at 100,000 *g* for 60 min. The band between 0.6 and 1.0 M sucrose (P_2B fraction) was collected. The pooled P_2B fraction was divided into aliquots and stored at −70°.

Before pretreatment with sulfhydryl-modifying reagents, the aliquots were diluted with 10 vol. of standard buffer (25 mM Tris–HCl pH 7.4 at 4°) and centrifuged at 25,000 *g* for 20 min, to obtain a pellet. The pellet was rehomogenized with standard buffer and incubated at 37° for 30 min to remove endogenous 5-HT. After the incubation, the suspension was recentrifuged at 25,000 *g* for 20 min, to obtain a pellet which was resuspended in fresh standard buffer for pretreatment with sulfhydryl-modifying reagents.

The membrane was "pretreated" as follows: it was preincubated in a standard medium consisting of 25 mM Tris–HCl buffer (pH 7.4) at 25° for 30 min in the presence or absence of PCP (10 μM), ritanserin (1 μM) or 5-HT (10 μM) and then incubated with an appropriate amount of sulfhydryl-modifying reagents at 25° for 30 min. The sufficient concentrations of PCP, ritanserin and 5-HT to occupy all of its binding sites were used according to the previous experiments [14, 15] and unpublished results. After the incubation with sulfhydryl-modifying reagents, the membrane was chilled in an ice-cold water bath and 2.5 mM dithiothreitol was added to the membrane, which was then isolated by centrifugation (25,000 *g* 20 min) at 4° to obtain a pellet. The pellet was rehomogenized with cold standard buffer and incubated at 37° for 30 min to remove PCP, ritanserin or 5-HT bound to the membrane. After the incubation, the suspension was recentrifuged (25,000 *g* 20 min), yielding a pellet which was resuspended in fresh standard buffer for the binding assay with tritiated ligand. [^{3}H]-ligand binding was initiated by the addition of 0.2 ml of the membrane preparation (containing approximately 0.2 mg of protein) to a mixture containing 1 nM (final concentration) of [^{3}H]PCP, [^{3}H]ketanserin and [^{3}H]5-HT in a total volume of 1.0 ml. Incubation was carried out at 25° for 30 min. The binding reaction was stopped by rapid filtering through a Whatman GF/B filter. The filter was washed twice with 5 ml of ice-cold standard buffer.

Specific binding was defined as the excess over blank containing 10 μM PCP, 5 μM methysergide and 10 μM 5-HT. For Scatchard plot, [^{3}H]PCP (0.1–20 nM) and [^{3}H]ketanserin (0.05–10 nM) was used for the binding assay and the Scatchard plots were divided into two independent components by the method of Rosenthal [27]. [^{3}H]PCP binding in the presence of 5 μM SKF 10,047, a sigma opiate receptor agonist, was determined to define the PCP receptors [28]. The 5-HT_2 receptors were defined by measuring [^{3}H]ketanserin binding in the presence of 5 μM prazosin, an $alpha_1$-adrenoceptor blocker [29, 30]. In the experiments concerning 5-HT binding, 10 μM pargyline, a monoamine oxidase (MAO) inhibitor, was added in the assay mixture.

The concentrations of site-selective displacing ligands were selected from an analysis of their potencies as displacers for each labeled ligand [14, 15]. Filters presoaked in 0.1% poly-L-lysine at 4° for 120 min were used for the binding experiments with [^{3}H]PCP to avoid "specific" ligand binding to

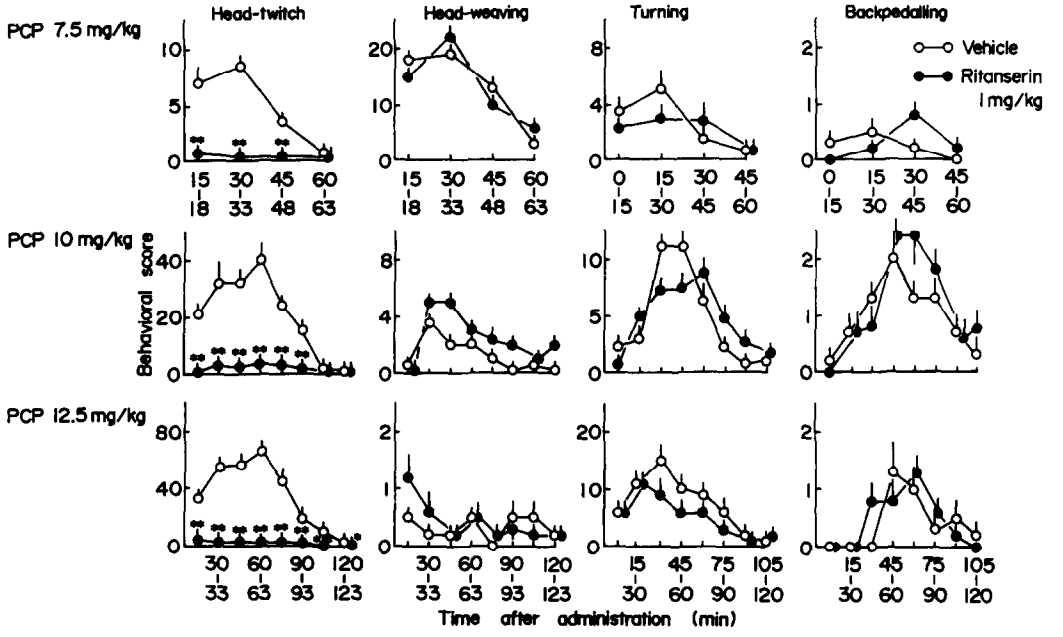

Fig. 1. Effect of pretreatment with ritanserin on the head-twitch induced by phencyclidine (PCP) in rats. Rats were given PCP (7.5, 10 and 12.5 mg/kg, i.p.) 1 hr after pretreatment with vehicle or ritanserin (1 mg/kg, s.c.). The behavioral rating scores were recorded as in Materials and Methods. Values are the means ± SE of 6–8 rats. $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle + PCP (Mann–Whitney U-test).

the filters [31]. The protein content of each membrane preparation was determined by the method of Lowry *et al.* [32].

Statistical analysis. All results were expressed as the means ± SE. Behavioral data were evaluated using the paired Mann–Whitney U-test. The results of binding assays were analyzed with the two-tailed Student *t*-test when the F values from the F-test were associated with $P > 0.05$ and with the Cochran *t*-test when those were associated with $P < 0.05$.

RESULTS

Effect of pretreatment with ritanserin on the PCP-induced behaviors

Head-twitch induced by PCP (7.5, 10 and 12.5 mg/kg, i.p.) was completely blocked by the pretreatment with ritanserin (1 mg/kg, s.c.), but head-weaving, turning and backpedalling induced by PCP were not blocked (Fig. 1).

Protective effects of PCP-pretreatment on inhibition of [^{3}H]PCP and [^{3}H]ketanserin bindings induced by NEM and IOAA

The effects of chemical modification of sulfhydryl groups on [^{3}H]PCP and [^{3}H]ketanserin bindings were examined by using NEM and IOAA. NEM and IOAA reduced the amount of binding of [^{3}H]PCP and [^{3}H]ketanserin in a concentration-dependent fashion (Figs 2, 3). PCP (10 μM) provided protection of [^{3}H]PCP and [^{3}H]ketanserin binding sites from the inactivation induced by NEM and IOAA (Figs 2, 3). Furthermore, the protective effect of PCP (10 μM) on [^{3}H]PCP and [^{3}H]ketanserin binding sites was equally effective against the inactivation induced by other sulfhydryl reagents such as *p*-chloromercuribenzoate and 5,5′-dithiobis(2-nitrobenzoic acid) (data not shown).

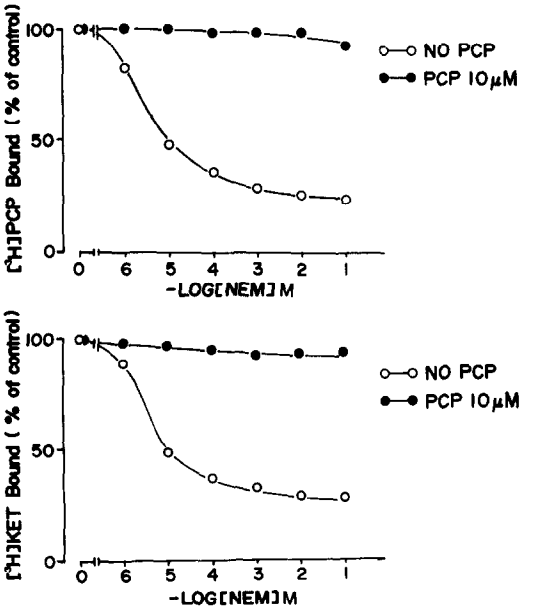

Fig. 2. Protective effects of PCP (10 μM)-pretreatment on inhibitions of [^{3}H]PCP and [^{3}H]ketanserin ([^{3}H]KET) bindings induced by *N*-ethylmaleimide (NEM). The amounts of [^{3}H]PCP and [^{3}H]ketanserin binding were 40.3 and 132.7 fmol/mg protein, respectively in the control group.

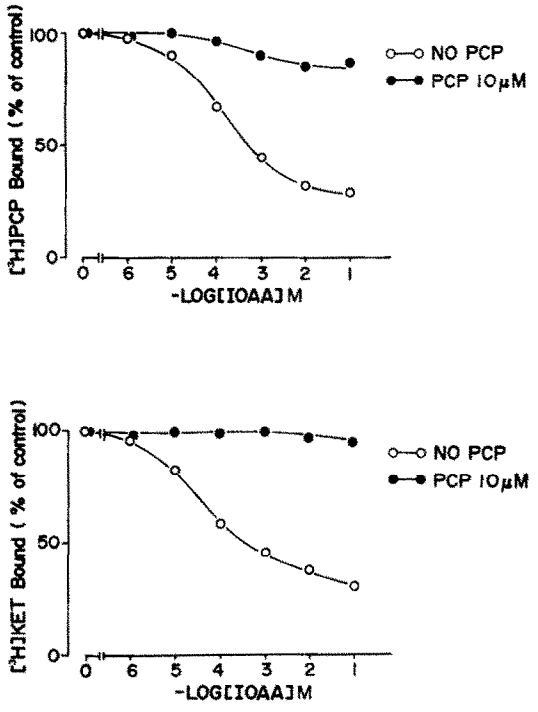

Fig. 3. Protective effects of PCP (10 μM)-pretreatment on inhibition of [³H]PCP and [³H]ketanserin ([³H]KET) bindings induced by iodoacetamide (IOAA). See legend for Fig. 2.

Effects of ritanserin- and 5-HT-pretreatments on inhibition of [³H]PCP, [³H]ketanserin and [³H]5-HT bindings induced by NEM

Ritanserin (1 μM) also completely prevented the inhibitory action of NEM on [³H]PCP and [³H]ketanserin bindings, but 5-HT (10 μM) did not (Fig. 4). Furthermore, as shown in Fig. 5, NEM also reduced the amount of binding of [³H]5-HT in a concentration-dependent fashion. 5-HT (10 μM) completely prevented the inhibitory action of NEM on [³H]5-HT binding, but PCP (10 μM) and ritanserin (1 μM) failed to protect it (Fig. 5).

Protective effects of PCP and ritanserin (RIT)-pretreatments on NEM-induced decrease of binding capacity in [³H]PCP and [³H]ketanserin binding sites

Scatchard plots of specific [³H]PCP and [³H]ketanserin bindings revealed the presence of two populations of binding sites (Fig. 6). Saturation studies of [³H]PCP and [³H]ketanserin bindings in the membrane pretreated with NEM (100 μM) showed a parallel shift in the Scatchard plot, with a reduction in the maximum number of binding sites but with no change in the apparent affinity. PCP (10 μM) and ritanserin (1 μM) provided protection of [³H]PCP and [³H]ketanserin binding sites from the decrease of the number of binding sites induced by NEM (100 μM) (Table 1). We got the high-affinity sites for PCP with $K_D = 0.27$ nM. This value is two-to-three orders of magnitude lower than the K_D of any PCP sites reported in the literature. The

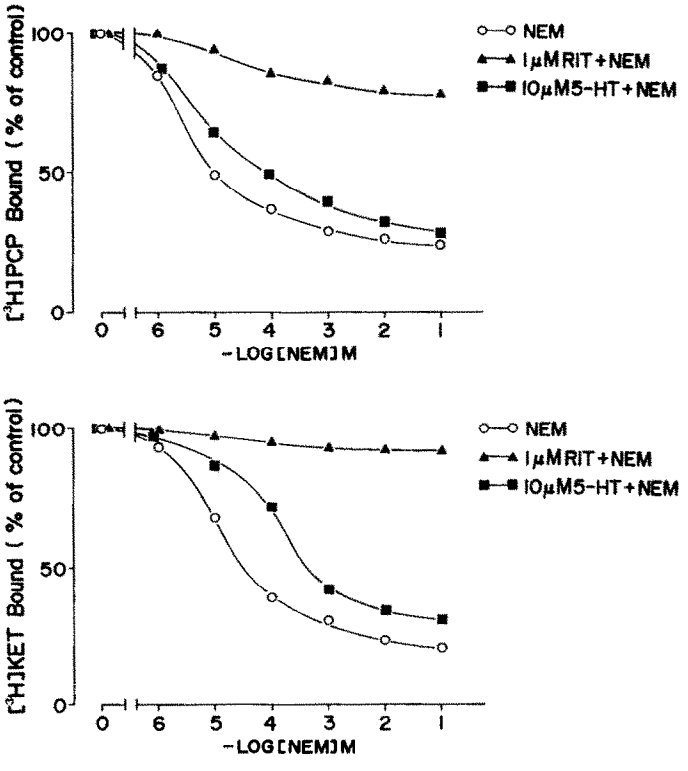

Fig. 4. Effects of ritanserin (RIT, 1 μM)- and 5-HT (10 μM)-pretreatments on inhibition of [³H]PCP and [³H]ketanserin ([³H]KET) bindings induced by *N*-ethylmaleimide (NEM). See legend for Fig. 2.

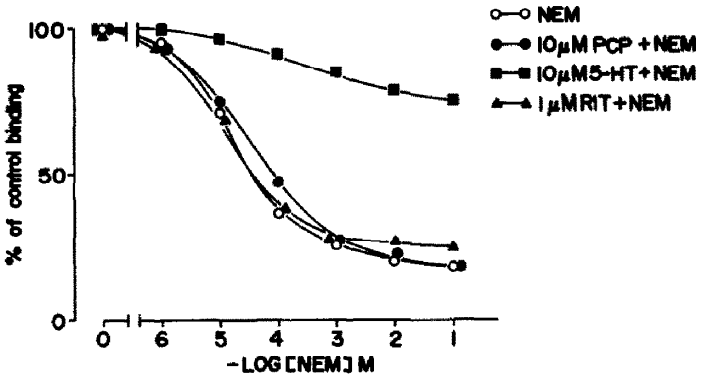

Fig. 5. Effects of PCP (10 μM)-, 5-HT (10 μM)- and ritanserin (RIT, 1 μM)-pretreatments on inhibition of [^{3}H] 5-HT binding induced by *N*-ethylmaleimide (NEM). The amount of [^{3}H] 5-HT binding was 186.5 fmol/mg protein in the control group

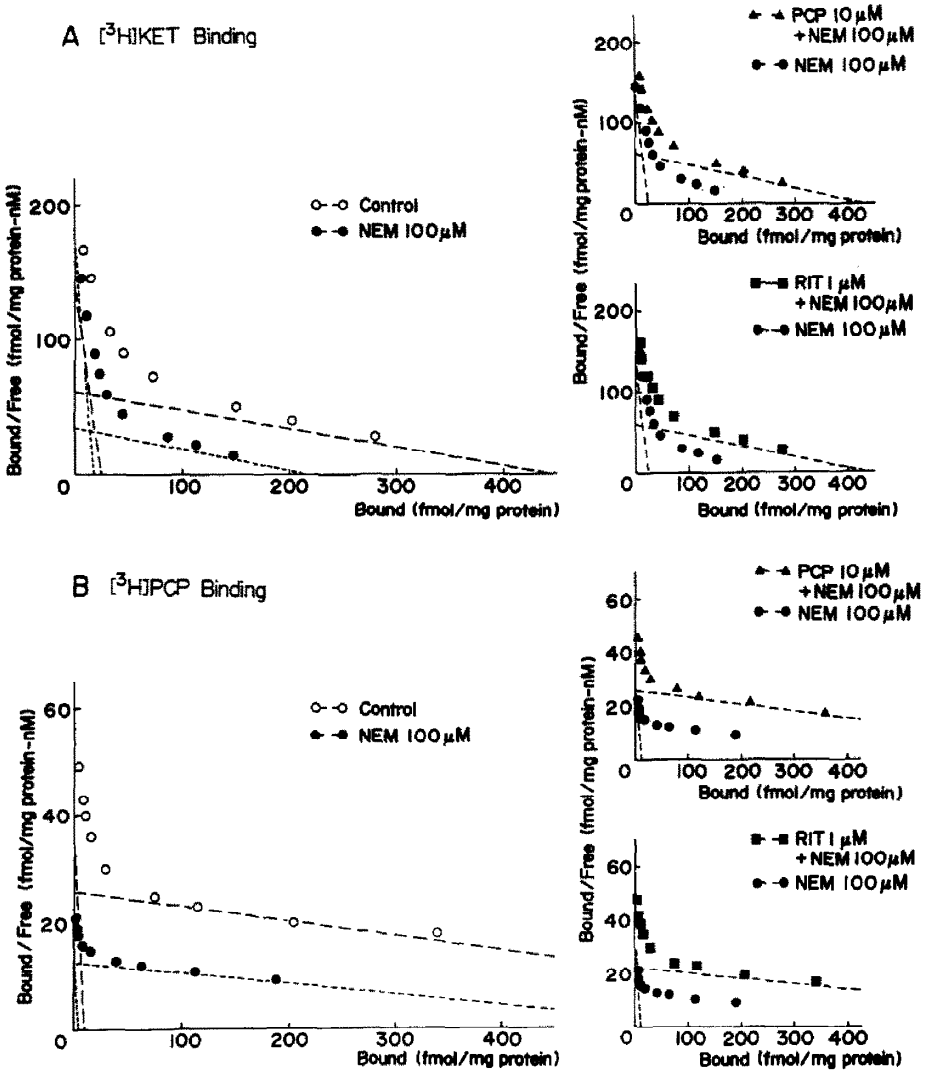

Fig. 6. Protective effects of PCP (10 μM)- and ritanserin (RIT 1 μM)-pretreatments on inhibition of [^{3}H]ketanserin ([^{3}H]KET, A) and [^{3}H]PCP (B) binding induced by *N*-ethylmaleimide (NEM).

Table 1. Protective effects of phencyclidine (PCP)- and ritanserin (RIT)-pretreatments on *N*-ethylmaleimide (NEM)-induced decrease of binding capacity in [^{3}H]PCP and [^{3}H]ketanserin binding sites

[^{3}H] Ligand	Treatments	Affinity	K_d (nM)	B_{max} (fmol/mg protein)
[^{3}H] PCP	Control	High	0.27 ± 0.03	9.6 ± 0.7
		Low	46.8 ± 2.4	1115.6 ± 55.3
	(100 μM)	High	0.26 ± 0.01	3.0 ± 0.4*
		Low	43.4 ± 1.6	562.5 ± 35.1*
	PCP (10 μM)	High	0.26 ± 0.03	7.3 ± 0.5*
	+ NEM (100 μM)	Low	42.4 ± 1.6	1085.8 ± 40.9†
	RIT (1 μM)	High	0.27 ± 0.02	8.8 ± 0.6†
	+ NEM (100 μM)	Low	47.2 ± 2.5	1126.8 ± 39.1†
[^{3}H] ketanserin	Control	High	0.15 ± 0.01	21.2 ± 0.5
		Low	6.92 ± 0.2	431.0 ± 13.6
	NEM (100 μM)	High	0.13 ± 0.02	16.8 ± 0.6*
		Low	6.12 ± 0.8	209.3 ± 2.3*
	PCP (10 μM)	High	0.15 ± 0.01	20.7 ± 0.2†
	+ NEM (100 μM)	Low	6.42 ± 0.3	412.2 ± 9.8†
	RIT (1 μM)	High	0.16 ± 0.01	22.5 ± 0.8†
	+ NEM (100 μM)	Low	7.0 ± 0.2	433.4 ± 6.8†

The values of dissociation constant (K_d) and maximum binding capacity (B_{max}) are the means of 3–4 independent determinations (± SEM), each in triplicate.
* $P < 0.01$ vs control, † $P < 0.01$ vs NEM (100 μM).

discrepancy between present results and the other author's data may be due to the fact that we used the synaptic membrane of P_2B fraction for binding assay, but the other author used crude P_2 fraction.

DISCUSSION

Some hallucinogens act as postsynaptic 5-HT receptor agonists [33], while others augment 5-HT synthesis and release [34]. As described in the introduction, administration of some hallucinogens to rats produces head-twitch which may be mediated via 5-HT_2 receptors.

The results of the present investigation demonstrated that PCP (one of the hallucinogens) induced head-twitch dose-dependently and that PCP-induced head-twitch was completely antagonized by ritanserin, a selective 5-HT_2 receptor antagonist. Therefore, the mechanism of PCP-induced head-twitch appears to involve an activation of the 5-HT_2 receptor. Furthermore, ritanserin failed to inhibit PCP-induced head-weaving, turning and backpedalling, although these behavioral changes are blocked by the non-specific 5-HT antagonist cyproheptadine [24]. These results suggest that PCP does not induce these behaviors by affecting 5-HT_2 receptors directly, and that PCP-induced head-weaving is mediated either via a 5-HT_{1A} recognition site since pindolol (5-HT_{1A} antagonist) antagonized this behavior [35, 36] and/or via some other mechanisms.

We have reported that PCP can displace [^{3}H]spiperone [14, 15] and [^{3}H]ketanserin (unpublished data) at the 5-HT_2 receptor sites, but not [^{3}H] 5-HT at the 5-HT_1 receptor sites [14, 15]. Furthermore, chronic administration of PCP produces down-regulation of 5-HT_2 [16] and PCP [37] receptors. Intensity of PCP-induced head-twitch and number of 5-HT_2 and PCP receptors are increased in the chronic methysergide-treated rats compared to the control rats [37]. From these results, we have suggested that PCP binding sites overlap 5-HT_2 receptors and PCP interacts directly with 5-HT_2 receptors.

The sulfhydryl groups play important roles in the function of receptors binding of their agonists, since sulfhydryl modifying reagents inactivate it [38, 39]. In general, selective agonists antagonize the inhibitory action of sulfhydryl modifying reagents on the specific receptors [38]. If PCP could interact with 5-HT_2 receptors selectively or allosterically, like ritanserin it could antagonize the inhibitory action of sulfhydryl modifying reagents on 5-HT_2 receptors, but not on 5-HT_1 receptors. If 5-HT_2 receptors were overlapped with PCP binding sites or there was an allosteric interaction between PCP and 5-HT_2 receptors, ritanserin as well as PCP could antagonize the inhibitory action of sulfhydryl modifying reagents on PCP binding sites. Proceeding from the above reports and hypotheses, we investigated whether an inhibitory effect of protein-modifying reagents (sulfhydryl blockers) on the bindings of [^{3}H]PCP and [^{3}H]ketanserin to 5-HT_2 receptors is prevented in the presence of PCP. The present results showed that PCP and ritanserin antagonized the decrease in the number of [^{3}H]PCP and [^{3}H]ketanserin binding sites induced by the sulfhydryl blocker. In contrast, the effects of the sulfhydryl blocker on [^{3}H]5-HT binding was blocked by the addition of 5-HT, but not by PCP and ritanserin. These results indicate that the protective effect of PCP is specifically due to a direct or allosteric interaction with 5-HT_2 receptors located at PCP binding sites in membranes.

On the basis of the present findings, it is concluded that PCP has an ability to perform as an agonist for 5-HT_2 receptors, and that PCP may interact with 5-HT_2 receptors directly or allosterically.

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