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Blockade of ACh Receptors by PrBCM Causes Deficits in Shuttle Avoidance Performance

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KATO, S., Y. KIMURA, N. MISAKI, M. NAKAHIRO AND H. YOSHIDA. *Blockade of ACh receptors by PrBCM causes deficits in shuttle avoidance performance.* PHARMACOL BIOCHEM BEHAV **33**(4) 895–898, 1989.—Studies were made examining the effect of blockade of muscarinic acetylcholine (mACh) receptors in the cerebral cortex of rats on their shuttle avoidance after training. Rats were given a session of shuttle avoidance tests once a day for 12 days. Then the irreversible antagonist of mACh receptors, propylbenzilylcholine mustard (PrBCM), was injected bilaterally into the cerebral cortex of rats showing avoidance rates of more than 75% in the last session, and avoidance rates were examined 24 hr later. The avoidance rates of the rats treated with 100 μg PrBCM were lower than those in the last session before treatment. The amount of mACh receptors in the cerebral cortex was decreased by PrBCM treatment, as shown by [^{3}H]quinuclidinyl benzilate (QNB) binding studies performed just after measurement of the avoidance response. The present study indicates that cholinergic neurotransmission in rat cerebral cortex is involved in performing a learned shuttle avoidance.

Cholinergic neurotransmission Conditioned avoidance Irreversible antagonist Muscarinic acetylcholine receptor Propylbenzilylcholine mustard (PrBCM)

CHOLINERGIC neurons in the central nervous system (CNS) are thought to be involved in learning and memory processes (4–6, 14). Disorder of these neurons is suspected to be a major cause of Alzheimer's disease, in which loss of memory and cognition is apparent (1, 3, 19). Blockade of cholinergic neurotransmission in the CNS is reported to disturb learning and memory in animals (9, 20, 21) and humans (7, 8, 15).

Propylbenzilylcholine mustard (PrBCM) is an alkylating antagonist for muscarinic acetylcholine (mACh) receptors. This drug has proved to be of high specificity and affinity and is a valuable tool in receptor pharmacology (2,22). Its pharmacologically active species is the aziridinium ion formed in aqueous solution and is presumed to bind covalently to the receptor (2). We have reported that blockade of cholinergic receptors in the rat cerebral cortex by this drug causes loss of memory of passive avoidance learning (10). However, a definite conclusion on the effect of a drug on learning and memory should not be drawn from results obtained by only one method (16). Conditioned avoidance tasks have been used to investigate learning and memory in rodents (11,16). Accordingly, in this work we used a shuttle-type conditioned avoidance test to investigate the effect of injection of PrBCM into the cerebral cortex of rats on learning and memory.

METHOD

Male Wistar rats (Charles River Japan, Inc.) weighing 200–240 g at the beginning of the training were used in this study. The procedures of the injection are the same as those described previously (10). Rats were anesthetized with Nembutal (50 mg/kg IP) and mounted in a David Kopf stereotaxic apparatus. A coronal incision was made in the scalp and holes were drilled through the skull in appropriate positions for injections. A solution of PrBCM at a dose of 20, 60 or 100 μg was injected into four loci in the brain (doses of 5, 15 and 25 μg in 3 μl of saline, respectively, or 3 μl of saline alone as a control were injected into each locus). These loci were bilateral in the frontal (A 8.0, L 2.8, V 1.0) and parietal (A 3.8, L 2.8, V 1.0) regions of the cortex according to the coordinates of Pellegrino and Cushman (13). Then the microsyringe was removed and the scalp was sutured.

PrBCM was converted to its active form just before use. In the previous work (10), we used the method of Burgen *et al.* (2) for the conversion, but in the present study we used the following

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TABLE 1

AVOIDANCE RATES IN THE 12TH SESSION AND THE SESSION AFTER PrBCM INJECTION

	Avoidance Rate (%)	
	12th Session	Session After Injection
Control	90.46 ± 7.83	88.89 ± 11.76 (9)
PrBCM		
20 μg	90.13 ± 6.92	87.86 ± 11.79 (7)
60 μg	90.83 ± 9.21	78.98 ± 26.12 (9)
100 μg	88.60 ± 8.84	57.41 ± 28.07* (9)

*Significantly different from the rate in the 12th session of the same group by Newman-Keuls test ($p<0.01$). Values are means ± S.D. for the numbers of rats shown in parentheses.

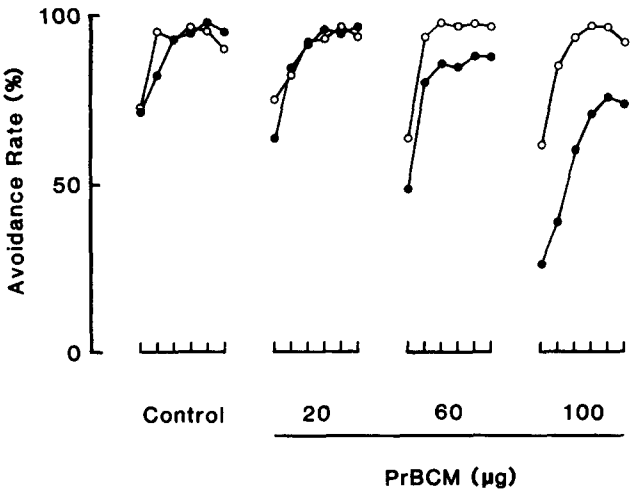

FIG. 1. Avoidance rates in the 12th session (○) and the session after PrBCM injection (●). Each session is divided into 6 parts. Points are means for 9 (control), 7 (20 μg PrBCM-treated), 9 (60 μg PrBCM-treated) and 9 (100 μg PrBCM-treated) rats.

procedure. PrBCM was dissolved in saline at an appropriate concentration and incubated at 37°C for 10 min. The solution was then cooled to 0–4°C and injected. As a control, saline at 0–4°C was injected.

The shuttle box (O'Hara & Co.) used for the avoidance task was made of acrylfiber board and its dimensions were 50 (W) × 15 (D) × 27 (H) cm. It had a metal plate of 3 cm height in the center and two photo-beams arranged 38.8 cm apart at both sides. A light for presenting a warning stimulus was set in the ceiling of the box. An electrical shock was applied through the metal grid of the floor. Shuttle locomotion of a rat from one side to the other was recorded by its interruption of the beams. The test rat was placed in one side of the box, and a warning light stimulus was applied from the lid of the box for 3 sec at intervals of 30 sec. If the rat did not move from one side of the box to the other by the end of the 3-sec warning stimulus, it received an electrical shock of 2 mA for 2 sec from the metal grid of the floor. If the rat moved to the other side during the warning, no foot-shock was applied and the response was counted as successful avoidance. If the rat moved to the other side during this foot-shock, the shock was stopped, but movement of the rat was not counted as successful avoidance. This trial was applied 120 times successively in each session every day. Accordingly, one session consisted of 120 trials. The avoidance rate was calculated as the percentage of successful avoidance in the trials. The number of shuttle locomotions during each trial was also recorded as the index of locomotor activity of rats, and this will be referred to as shuttle locomotions per trial in this report.

Rats showing an avoidance rate of more than 75% in the 12th session were treated with PrBCM 24 hr later, while rats showing lower avoidance rates were excluded from the test. The rats treated with PrBCM were tested again for shuttle avoidance 24 hr after the injection. Then they were promptly decapitated and mACh receptors of the regions treated with PrBCM were measured by [^{3}H]QNB binding assay as described previously (10,12).

PrBCM was a gift from Tanabe-Seiyasku Co. [^{3}H]QNB (16.0 Ci/mmol) was purchased from Amersham.

RESULTS

The avoidance rate of the rats used for analysis increased progressively session by session. It was observed that rats showed higher avoidance rates in the later part than the earlier part of each session. We refer to this phenomenon as "warming-up" in this report.

In the avoidance rates, there was a significant interaction of time (before and after PrBCM injection) and PrBCM doses with mixed two-factor ANOVA, $F(3,60)=3.27$, $p<0.05$. Post hoc test (Newman-Keuls), moreover, indicated significant differences between before and after injection for 100 μg PrBCM-treated group ($p<0.01$, Table 1).

The avoidance rates in the 12th session and the session after the injection are shown in Fig. 1, where each session was divided into 6 parts of 20 trials each and each point represents the avoidance rate in these 20 trials. This figure clearly shows the warming-up phenomenon in both sessions of each group. The results show that after injection of 60–100 μg PrBCM, the avoidance rates decreased to approximately the same extent in each part of the session, although after injection of 60 μg of PrBCM, the decrease in the avoidance rate throughout the session was not statistically significant (Table 1).

PrBCM concomitantly decreased the amount of mACh receptors dose-dependently as shown by [^{3}H]QNB binding assay. The significance of irreversible antagonism by PrBCM was confirmed with the percent decrease data. The overall group effect was significant, $F(3,22)=27.29$, $p<0.01$, and the additional Newman-Keuls tests revealed that all of the PrBCM-treated groups differed significantly from the control ($p<0.01$ in all pairs) (Table 2).

Table 3 shows shuttle locomotions in the 12th session and the session after PrBCM injection. There was no significant interaction, $F(3,60)=0.321$, $p>0.05$, or main effects, $F(1,60)=0.013$, $p>0.05$; $F(3,60)=0.97$, $p>0.05$, with mixed two-factor ANOVA.

DISCUSSION

The "warming-up" phenomenon was reported in other conditioned avoidance tests (11). In the present study, because of this phenomenon, the avoidance rates were lower in earlier parts of a session than in later parts of the preceding session. However, the average avoidance rate in each session was higher than that in the preceding session until these rates reached a level of more than 75%. Furthermore, this high avoidance rate could be maintained in rats by one session a day. Thus, in this study, rats that acquired an avoidance rate of more than 75% were used for analysis.

In the previous study, we observed that PrBCM injection into the frontal and/or parietal regions of the cortex, but not into the occipital region of the cortex, caused loss of ability to perform a passive avoidance task (10). Therefore, in this study, PrBCM was

TABLE 2

EFFECT OF PrBCM INJECTION ON THE AMOUNT OF [^{3}H]QNB BINDING SITES

	[^{3}H]QNB Binding Sites pmol/mg Protein	%
Control	1.78 ± 0.19 (7)	100
PrBCM		
20 μg	0.86 ± 0.07* (5)	48
60 μg	0.52 ± 0.02* (7)	29
100 μg	0.42 ± 0.01* (7)	24

*Significantly different from the control by Neuman-Keuls test ($p<0.01$). Values are means ± S.D. for the numbers of the rats shown in parentheses. Percentages of the control value are indicated in the right column.

TABLE 3

SHUTTLE LOCOMOTIONS IN THE 12TH SESSION AND THE SESSION AFTER PrBCM INJECTION

	Shuttle Locomotions per Trial	
	12th Session	Session After Injection
Control	1.22 ± 0.26	1.19 ± 0.29 (9)
PrBCM		
20 μg	1.33 ± 0.32	1.38 ± 0.30 (7)
60 μg	1.35 ± 0.39	1.24 ± 0.39 (9)
100 μg	1.33 ± 0.32	1.48 ± 0.51 (9)

The average shuttle locomotion in one trial was calculated and is indicated as the shuttle locomotion per trial. Values are means ± S.D. for the numbers of rats shown in parentheses.

injected into both the frontal and parietal regions of the cortex.

Changes in locomotor activity and susceptibility to foot-shock could affect results on shuttle avoidance in this study. However, as reported previously, PrBCM injection does not affect locomotor activity or susceptibility to foot-shock (10). Moreover, the fact that the shuttle locomotions per trial were not significantly different before and after the injection (Table 3) confirms that PrBCM injection does not affect the locomotor activity of rats.

Although PrBCM is an irreversible antagonist in short-term tests (2,20), its blockade of mACh receptors has a half-life of about 3 days (10). Judged from this long half-life, the [^{3}H]QNB binding sites represented mACh receptors during the last session. PrBCM decreased mACh receptors at doses of 20–100 μg. No behavioral deficit was apparent at 20 μg PrBCM, although receptors decreased to 48% of the control. But 100 μg PrBCM was necessary for significant lowering of the avoidance rate. This dose decreased mACh receptors to approximately one-quarter of the quantity in controls. Thus, it seems likely that a part of the receptors is not necessary for performing the shuttle avoidance task. It has been reported that the maximal contraction of a smooth muscle can be obtained even after treatment of the muscle with PrBCM (18). Takeyasu *et al.* have also reported that in guinea pig and mouse ileum only a portion of the receptors is required to be occupied to produce a maximal response and the receptors remaining unoccupied during the maximal response have been designated as "spare receptors" (17). The results in this study suggest that cholinergic system has such spare receptors in the CNS as well as in smooth muscles.

Figure 1 further shows the effects of PrBCM treatment on "warming-up." Although the avoidance rate throughout the session was reduced by 60–100 μg PrBCM, the warming-up itself was similar before and after PrBCM injection. It is interesting that blockade of mACh receptors did not affect this phenomenon.

It has been reported that blockade by PrBCM of mACh receptors in the frontal and parietal regions of the cortex of rats causes loss of ability to perform a passive avoidance task (10). The present results indicate that the same treatment impairs ability to perform a learned shuttle avoidance.

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