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Loss of anti-cataleptic effect of scopolamine in mice lacking muscarinic acetylcholine receptor subtype 4

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Abstract

Motor dysfunction associated with dyskinesia can be caused by imbalance between dopaminergic and cholinergic actions. Antimuscarinic agents are used to treat extrapyramidal symptoms in Parkinson's disease and extrapyramidal side effects of antipsychotics. These therapeutic effects are mediated by blockade of the striatal muscarinic receptors, which comprise five distinct subtypes (M_{1-5}). To evaluate the role of muscarinic M_4 receptors, we have generated mutant mice lacking this subtype (muscarinic M_4 receptor-knockout mice) and analyzed their cataleptic responses induced by haloperidol (an animal model of extrapyramidal side effects). While the muscarinic M_4 receptor-knockout mice developed the cataleptic response normally, systemic administration of scopolamine could not suppress the cataleptic response. These results suggest that acute, but not chronic, blockade of muscarinic M_4 receptors plays important roles in the therapeutic effects of antimuscarinic agents.

Keywords: Muscarinic receptor; Catalepsy; Extrapyramidal side effect; Haloperidol; Scopolamine; Knockout mouse

1. Introduction

It has been recognized for many years that balance between the dopaminergic and cholinergic actions in the striatum plays a key regulatory role in the extrapyramidal motor control. The central-acting muscarinic receptor antagonists have been used to treat extrapyramidal movement disorders, such as Parkinson's disease and antipsychotics-induced parkinsonism. Among the five subtypes of muscarinic acetylcholine receptors (Caulfield and Birdsall, 1998), muscarinic M_1 and M_4 receptors are expressed abundantly in the striatum (Hersch et al., 1994). In addition, muscarinic M_4 receptors and dopamine D1 receptors colocalize on most striatonigral neurons, and appear to regulate the cAMP levels in a reciprocal manner (Ince et al., 1997). Thus, it has been suggested that muscarinic M_4 receptors are most likely involved in the striatal motor control. However, lack of specific ligands for each subtype has hampered further detailed studies.

To elucidate the role of muscarinic M_4 receptors, we have generated a mutant mouse line deficient in this subtype (muscarinic M_4 receptor-knockout mice). Here, we have performed behavioral tests, including haloperidol-induced catalepsy (Hoffman and Donovan, 1995), which is one of the animal models for antipsychotic-induced parkinsonism. We have tested the knockout mice after backcrossing into the DBA/2J strain, which is susceptible to extrapyramidal side effects of haloperidol (Kanes et al., 1993).

2. Materials and methods

2.1. Gene targeting

The genomic cloning of a muscarinic M_4 receptor gene (*Chrm4*) from a mouse 129/SvJ library was described previously (Matsui et al., 1999). A targeting vector, pChrm4-N (Fig. 1A), was constructed from the inserts of

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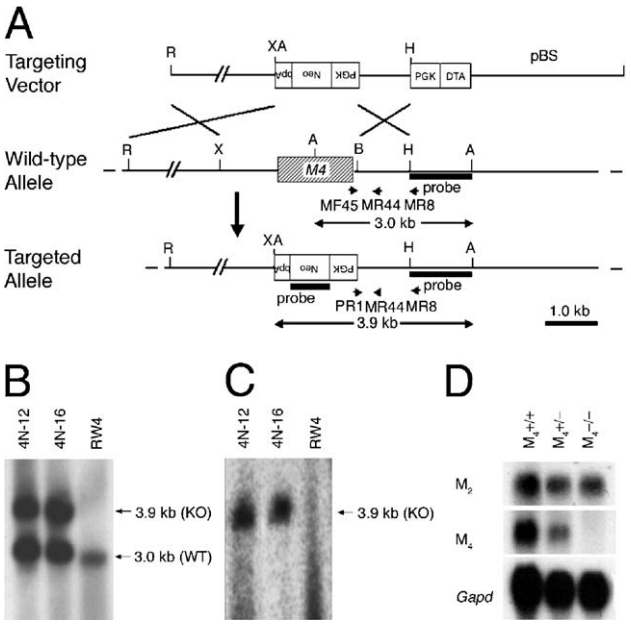

Fig. 1. Generation of muscarinic M_4 receptor-knockout ($M_4-/-$) mice. (A) Targeting strategy. Arrows PR1 and MR8 indicate the PCR primers used for homologous recombinant screening, whereas MF45, MR44 and PR1 for genotyping. *Apa*I (A), *Bgl*II (B), *Hin*dIII (H), *Eco*RI (R) and *Xba*I (X) sites relevant to the identification of homologous recombinant clones are shown together with the expected sizes hybridizable to the M_4 and the *neo* probes. (B) Hybridization with the M_4 probe showing a 3.9-kb band specific to the targeted allele and a 3.0-kb band derived from the wild-type allele. (C) Hybridization with the *neo* probe showing a 3.9-kb band specific to the targeted allele. (D) Northern blot analysis showing mRNA levels of muscarinic M_2 and M_4 receptors in the brains of $M_4+/+$, $M_4+/-$, and $M_4-/-$ mice. Note that the mRNA of muscarinic M_4 receptors in the $M_4+/-$ brain decreased to about half of the $M_4+/+$ brain and absent in the $M_4-/-$ brain. The mRNA levels of muscarinic M_2 receptors are not different among the three genotypes. The lowest panel shows the signals hybridized with a *Gapd* probe used as an internal control.

the phage clones. The *Hin*dIII–*Bgl*II (1.1 kb) and *Xba*I–*Eco*RI (9.6 kb) fragments were placed upstream and downstream of the phosphoglycerate kinase I promoter (PGK)-neo-bpA cassette (Soriano et al., 1991), respectively. The PGK-DTA cassette (Yagi et al., 1990) was inserted at the upstream end in the reverse orientation.

ES cells (RW4, Genome Systems) were electroporated with the targeting vector linearized at the unique *Sal*I site. G418-resistant clones were screened by polymerase chain reaction (PCR) with primers MR8 (5′-TGT TCT CCC CAC CCT GAT AC-3′) and PR1 (5′-CAG ACT GCC TTG GGA AAA GC-3′) to amplify a 1.2-kb fragment. Homologous recombination of the candidate clones was further verified by Southern hybridizations (Fig. 1B and C). Mutant mice harboring the mutant allele were generated as described (Matsui et al., 2000). Genotype was determined using PCR with the following primers: MF45 (5′-GTG CTG GTG TCA AGA GTG TG-3′), PR1 (5′-CAG ACT GCC TTG GGA AAA GC-3′) and MR44 (5′-GGA AGT CCC TCA CCT TGG AG-3′).

2.2. Northern blot analysis

Northern blot analysis was performed as described (Matsui et al., 2000) using total RNA extracted from the whole brain (except for the cerebellum) of 4-month old male mice. As a template of a *Chrm2* probe, a cloned 0.51-kb *Sma*I–*Sma*I genomic fragment was used. For *Chrm4* and *Gapd* probes, 0.35- and 0.50-kb DNA fragments, respectively, were amplified with PCR from C57BL/6J genomic DNA using the following primers: for *Chrm4*, Chrm4F (5′-AGC CGC AGC CGT GTT CAC AA-3′) and Chrm4R (5′-TGG GTT GAG GGT TCG TGG CT-3′); for *Gapd*, GapdF (5′-GCG TCC TGC ACC AAC TG-3′) and GapdR (5′-ATG GTC CTT TAC TCG AAG TG-3′).

2.3. Behavioral studies

Animals had been backcrossed to a DBA/2J strain four times (N4 backcross generation) and were between 3 and 6 months old. The mice used in this study consist of six wild-type males, five wild-type females, six knockout males, and three knockout females. They were group-housed (three to six animals per cage) in a temperature- and humidity-controlled vivarium under a 12 h-light/12 h-dark cycle in a specific pathogen-free area. Experiments were carried out with mice of both sex because we found no sex difference in a pilot study (data not shown).

Seven days before the behavioral test, a sham test was performed to assure that the animals did not develop cataleptic responses to saline injection. None of the mice showed any sign of cataleptic responses. Ascending dose of haloperidol alone or haloperidol and scopolamine was administered with at least 1 week of interval. After a habituation for 30 min in a test cage (13 cm height × 15 cm × 10 cm, Plexiglas), haloperidol (0.1 or 0.5 mg/kg, i.p.) alone or haloperidol (0.5 mg/kg, i.p.) and scopolamine (10 mg/kg, s.c.) were administered. Thirty minutes after the drug administration, cataleptic responses were assessed by a horizontal bar test (Saga et al., 1999). Briefly, both of the front limbs of the mice were hooked on the horizontal bar (2-mm diameter, stainless steel). The height of the bar was lower in females than in males (4.0 cm in females and 4.5 cm in males), which allowed similar standing posture of all animals tested. Then, the duration of this unusual posture was measured up to 180 s. Gross behavioral changes were assessed for 5 min just before the bar test by scoring as follows: 0, no locomotor change, walking normally; 1, moderate suppression of locomotor activity, reduced walking; 2, complete suppression of locomotor activity, still in the corner of the cage.

All procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

2.4. Drugs

The following drugs were used: (–)-scopolamine hydrobromide (Research Biochemicals) and haloperidol (Serenace; Dainippon Pharmaceutical). Scopolamine was dissolved in saline and injected subcutaneously in a volume of 10 ml/kg. Haloperidol was diluted in saline and injected intraperitoneally in a volume of 10 ml/kg.

2.5. Statistical analysis

Cataleptic response and general behavioral suppression were analyzed using *t* test and Wilcoxon test, respectively.

3. Results

3.1. Generation of muscarinic M_4 receptor-knockout mice

Genotype distribution of the pups born from intercrosses was approximately at the Mendelian ratio (+/+:+/−:−/− = 51:75:41). They appeared healthy and their general behavior in their home cage was normal. Histology of major organs including the brain and blood chemistry data were normal. Northern blot analysis revealed that the muscarinic M_4 receptor mRNA was absent in the homozygous brain, and was reduced to about half level in the heterozygous brain (Fig. 1D). No compensatory change in the amount of muscarinic M_2 receptor mRNA was detected. These findings are consistent with a previous report of another muscarinic M_4 receptor-deficient mouse line (Gomeza et al., 1999). In this report, the M_4 receptor-deficient mice were found to be hyperactive than wild-type control in an open field test and showed greatly enhanced locomotor responses after activation of D1 receptors. In our preliminary experiments, the basal locomotor activity of the M_4 receptor-knockout mice was not significantly different from that of the wild-type controls. However, enhancement of locomotor activity by a D1 receptor-selective agonist (SKF81297) was significantly stronger in the M_4 receptor-knockout mice than in the wild-type control (data not shown).

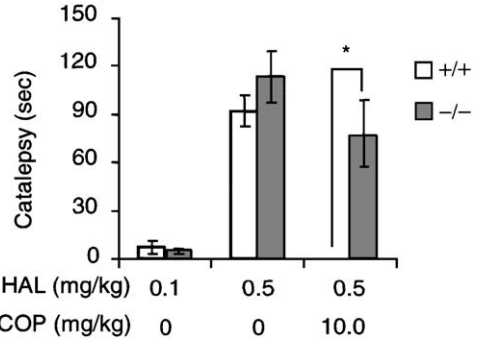

Fig. 2. Cataleptic response induced by haloperidol (HAL), and inhibitory effect of scopolamine (SCOP) on the catalepsy. Data are given as the mean ± S.E.M. ($n = 11$ for wild type and 9 for knockout). *$P < 0.01$ (*t* test).

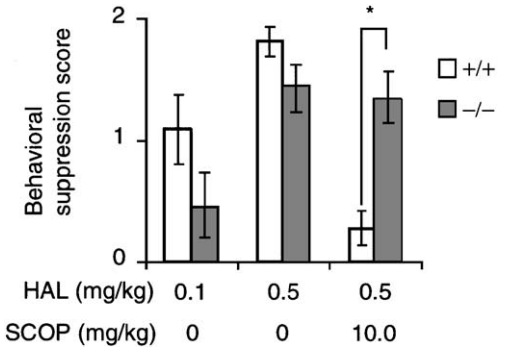

Fig. 3. Suppression of gross behavior by haloperidol (HAL), and inhibitory effect of scopolamine (SCOP) on the behavioral suppression. Data are given as the mean ± S.E.M. ($n = 11$ for wild type and 9 for knockout). *$P < 0.05$ (Wilcoxon test).

3.2. Haloperidol-induced catalepsy

The durations of catalepsy caused by 0.1 mg/kg of haloperidol in the wild-type and knockout mice were 7.5 ± 4.2 and 5.0 ± 1.3, respectively (mean ± S.E.M., $P = 0.62$). When 0.5 mg/kg of haloperidol was used, the durations were increased to 92.1 ± 10.2 and 113.6 ± 16.1 ($P = 0.26$). Thus, the cataleptic responses to haloperidol were essentially identical between the wild-type and the knockout mice (Fig. 2). However, the responses to muscarinic receptor antagonist scopolamine (10 mg/kg) were different between the genotypes. Consistent with a previous report (Haraguchi et al., 1997), scopolamine (10 mg/kg) abolished haloperidol-induced catalepsy in the wild-type mice. In contrast, in the M_4 receptor-knockout mice, haloperidol-induced catalepsy was only weakly affected by co-administration of scopolamine (76.4 ± 22.4; $P = 0.0013$, compared with the wild type). Thus, the anti-cataleptic effect of scopolamine was virtually absent in the muscarinic M_4 receptor-knockout mice.

3.3. Behavioral suppression by haloperidol

We assessed the degree of suppression of gross behavior and exploration caused by haloperidol just before performing the bar test. When 0.1 mg/kg of haloperidol was administered, the behavioral suppression scores in the wild-type (1.1 ± 0.28) and knockout (0.44 ± 0.29) mice were not different significantly ($P = 0.11$). When 0.5 mg/kg of haloperidol was used, the scores were increased to 1.8 ± 0.12 and 1.4 ± 0.18, respectively, and there was no statistical difference between the genotypes ($P = 0.089$). Thus, the behavioral suppression by haloperidol was not altered by the ablation of the muscarinic M_4 receptors (Fig. 3). As reported (Shannon and Peters, 1990), co-administration of scopolamine blocked the haloperidol-induced behav-

ioral suppression in the wild-type mice (0.27 ± 0.14). In contrast, the behavioral suppression of the knockout mice could not be blocked by scopolamine (1.3 ± 0.23; $P = 0.013$, compared with wild type). Thus, the anti-behavioral suppression effect of scopolamine was also abolished in the muscarinic M_4 receptor-knockout mice.

4. Discussion

Muscarinic receptor agonists enhance haloperidol-induced catalepsy, while muscarinic receptor antagonists inhibit catalepsy (Klemm, 1985). However, the precise mechanism of these muscarinic actions remains unclear. Among the five subtypes of muscarinic receptors, muscarinic M_4 receptors have been suggested to play important roles in the striatal motor control. In pharmacological binding assay, 45% of the muscarinic binding site in the striatum showed the property of M_4 (Waelbroeck et al., 1990). An immunoprecipitation study also revealed that 45% of the striatal muscarinic receptor was M_4 (Yasuda et al., 1993). Using immunohistochemistry, the expression of muscarinic M_4 receptors was detected in 92% of the striatonigral neurons (Ince et al., 1997). In this report, it was also shown that most of the striatonigral neurons co-express muscarinic M_4 receptors and dopamine D1 receptors. Because these two receptors play opposing roles in the cAMP metabolism (i.e., stimulation of the dopamine D1 receptors activates adenylyl cyclase via the G_s protein, whereas stimulation of the muscarinic M_4 receptors inhibits the enzyme activity via G_i), it was suggested that the "acetylcholine–dopamine balance" reflects the counteracting effects of these neurotransmitters on the cAMP levels. To understand the significance of muscarinic M_4 receptors, we have evaluated the cataleptic response in the mutant mice lacking this subtype.

Unexpectedly, we did not detect any difference in the cataleptic responses between the muscarinic M_4 receptor-knockout mice and the wild-type mice. Interestingly, however, co-administration of scopolamine to the knockout mice failed to block their cataleptic responses. Therefore, it is suggested that the anti-cataleptic effect of antimuscarinic agents is dependent upon acute blockade of the muscarinic M_4 receptor-mediated signaling. This interpretation is consistent with the pharmacological finding that therapeutic efficacy of anticholinergics on Parkinson's disease correlates well with their potencies to inhibit adenylyl cyclase activity (Olianas and Onali, 1996). It should be also noted that co-administration of scopolamine did not reverse the haloperidol-induced behavioral suppression in the knockout mice. It remains to be determined whether this phenomenon is associated with the loss of anti-cataleptic effect.

Under our experimental conditions, the cataleptic responses in the knockout mice were indistinguishable from those in the wild-type mice. Therefore, it is reasonable to conclude that chronic loss of the signaling mediated by muscarinic M_4 receptors does not affect the haloperidol-induced catalepsy significantly. The result may reflect a chronic adaptation of the nervous system in the knockout mice. Although muscarinic M_2 receptors share the intracellular signaling mechanism with muscarinic M_4 receptors, it is unlikely that the former compensates for the loss of the latter. If muscarinic M_2 receptors play an essential role in the haloperidol-induced catalepsy instead of muscarinic M_4 receptors, the nonselective muscarinic receptor antagonist, scopolamine, should be effective to suppress the catalepsy in the muscarinic M_4 receptor-knockout mice. Rather, the compensation may involve alterations in the non-muscarinic signaling pathways. For example, signaling through the dopamine D1 receptor may be downregulated as an adaptation for the chronic ablation of the muscarinic M_4 receptor-mediated signals.

The sensitivity to haloperidol-induced catalepsy is known to vary among the mouse strains. For example, the ED_{50} values in DBA/2J and C57BL/6J strains were reported to be 0.45 and 3.9 mg/kg, respectively (Kanes et al., 1993). The dose response we observed was consistent with these values. In various behavioral tests, choosing an appropriate genetic background for a mouse strain is critical (Crawley et al., 1997). We have also tested the muscarinic M_4 receptor-knockout mice in the C57BL/6J background (N3) and found that a much higher dose of haloperidol (20 mg/kg) was required to keep them in the unusual posture in the bar test. Under such conditions, scopolamine did not release the mice from the unusual posture even in the wild type. It is likely that such a high dose of haloperidol resulted in neuroleptic effects rather than catalepsy. Therefore, it was practically impossible to analyze the effect of scopolamine on haloperidol-induced catalepsy in a C57BL/6J background.

In conclusion, acute, but not chronic, ablation of muscarinic M_4 receptor-mediated signaling is supposed to be the basis of anti-cataleptic effect of muscarinic receptor antagonists. We propose that specific antagonist for muscarinic M_4 receptor may be efficacious in treating the extrapyramidal symptoms in Parkinson's disease and drug-induced parkinsonism, with more clinical potency and less side effects.

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