Motor Dysfunction in Type 5 Adenylyl Cyclase-null Mice*

Received for publication, February 14, 2003, and in revised form, March 12, 2003

Published, JBC Papers in Press, March 28, 2003, DOI 10.1074/jbc.C300075200

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Various neurotransmitters, such as dopamine, stimulate adenylyl cyclase to produce cAMP, which regulates neuronal functions. Genetic disruption of the type 5 adenylyl cyclase isoform led to a major loss of adenylyl cyclase activity in a striatum-specific manner with a small increase in the expression of a few other adenylyl cyclase isoforms. D1 dopaminergic agonist-stimulated adenylyl cyclase activity was attenuated, and this was accompanied by a decrease in the expression of the D1 dopaminergic receptor and Gαi2. D2 dopaminergic agonist-mediated inhibition of adenylyl cyclase activity was also blunted. Type 5 adenylyl cyclase-null mice exhibited Parkinsonian-like motor dysfunction, i.e. abnormal coordination and bradykinesia detected by Rotarod and pole test, respectively, and to a lesser extent locomotor impairment was detected by open field tests. Selective D1 or D2 dopaminergic stimulation improved some of these disorders in this mouse model, suggesting the partial compensation of each dopaminergic receptor signal through the stimulation of remnant adenylyl cyclase isoforms. These findings extend our knowledge of the role of an effector enzyme isoform in regulating receptor signaling and neuronal functions and imply that this isoform provides a site of convergence of both D1 and D2 dopaminergic signals and balances various motor functions.

EXPERIMENTAL PROCEDURES

Generation of Knockout Mice—We disrupted the AC5 gene by the homologous recombination technique at the exon with the first translation initiation site (Fig. 1A). The type 5 AC gene has another translation initiation site with a reasonable Kozak consensus sequence within the same exon (8) that was excised in the final targeting vector. The integration of the knockout transgene was confirmed by genomic Southern analysis (Fig. 1B). All mice were 129/SvJ-C57BL/6 mixed background littermates from F1 heterozygote crosses. All experiments were performed in 8–12-week-old homozygous (AC5/−/−) and wild-type (WT) littermates. This study was approved by the Animal Care and Use Committee at the Yokohama City University School of Medicine and New Jersey Medical School.

RNase Protection Assay—Partial fragments of mouse AC cDNA clones for each isoform (types 1–9) and neuropeptides, i.e. enkephalin, substance P, and dynorphin, were obtained by PCR. A human 28 S ribosomal RNA probe was used as an internal control. RNase protection assay was performed using the RPA III kit (Ambion, Austin, TX).

AC Assay—Striatal tissues were dissected from mice, and membrane preparations were prepared for AC assays as described previously (10, 11).

Radioligand Binding Assay—D1 and D2 dopaminergic receptor binding assays were performed using [3H]SCH23390 and [3H]spiperone, respectively, as described previously (12, 13). Preliminary experiments demonstrated that the Kd and Bmax values for D1 and D2 dopaminergic receptors were similar to those reported previously (12, 13).

Behavioral Tests—Motor functions of mice were assessed by Rotarod test (14), locomotor activity tests (14, 15), pole test (16), and tail suspension test (17).

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Fig. 1. Gene targeting strategy and characterization of AC5−/− mice. The type 5 AC was disrupted by homologous recombination technique at the 5'-end of the type 5 AC gene containing the exon with the first translation initiation site. The type 5 AC gene has an additional downstream translational start site accompanied by a reasonable Kozak consensus sequence within the same exon that was excised in the targeting vector (A). A Southern blot of the targeted allele of tail DNA of F2 mice is shown in B. KO, knockout; K, KpnI; B, EcoRI; X, XhoI; A, Apal; P, PatI; BS, BosHII; H, HindIII; RV, EcoRV; N, Ncol; B, BamHII.

Fig. 2. Motor dysfunction in AC5−/− mice. A, Rotarod test. Each mouse was placed on a 3.5-cm-diameter rod covered with rubber to evaluate Rotarod performance (14). Mice were left for 1 min on the rod for habituation. The rod rotated gradually increasing from 4 to 40 rpm over the course of 5 min, and the time that mice could stay on an accelerating Rotarod without failing was recorded. Five trials were conducted for each individual 10–25 min apart within the dark phase of the light/dark cycle. Mice that stayed on the Rotarod for >300 s were considered complete responders, and their latencies were recorded as 300 s. *, p < 0.05 relative to +/+ ; +, p < 0.05 relative to +/− ; n = 15–20. B, pole test. To evaluate bradykinesia, pole test was performed (16). In brief, mice were placed head upward on the top of a rough surfaced pole (8 mm in diameter and 50 cm in height) that was wrapped with gauze to prevent slipping. The time until the mouse turned completely downward (open bars, Tturn) and the time it climbed down to the floor (closed bars, Tdesc) were measured. *, p < 0.01 relative to +/+ ; n = 14–17. Means ± S.E. are shown. Homozygous (AC5−/−) (closed circles, −/−), heterozygous (shaded circles, +/−), and WT (open circles, +/+) are shown.

Fig. 3. AC activity and its mRNA expression in various brain regions. AC catalytic activity was compared as described previously (15) using the membrane preparations from the striatum, cortex, and cerebellum (A). AC assays were conducted in the presence of 50 μM forskolin. Expression of AC5 mRNA was quantitated by RNase protection assays with 28S rRNA as loading standard (B). A representative result is shown. D1 or D2 receptor agonist-stimulated AC activity was also examined (C). AC assays were conducted in the presence of 1 μM SKF38393 (SKF) or 1 μM SKF38393 and 10 μM quinpirole (QPL) in a reaction buffer containing 10 μM GTP. Each AC catalytic activity was compared with that in the presence of 10 μM GTP alone. Open bars, WT (+/+); closed bars, AC5−/− (−/−). Means ± S.E. are shown. *, p < 0.05; **, p < 0.01 relative to +/+ ; n = 4. NS, not significant.
RESULTS

Impaired Motor Functions—Given that AC is the major effector enzyme of dopaminergic signals in the striatum, we conducted various motor function tests to evaluate striatal function in an animal model in which the AC5 gene was disrupted (AC5−/−). The most dramatic change was found in coordinated movement, which was evaluated by Rotarod performance. In this test, we measured the time that mice could stay on an accelerating Rotarod without falling. In general, there was a major impairment in AC5−/− and to a lesser degree in the heterozygous mice relative to WT (Fig. 2A). AC5−/− could spend significantly less time on the Rotarod, and heterozygous mice could spend slightly less time than WT. When tests were repeated, their performance improved significantly after a few trials. However, AC5−/− performed very poorly even after several trials. When the test was repeated on the following day, the results were similar, disputing the possibility that AC5−/− required a longer period to learn the performance (data not shown).

Spontaneous activity was determined both horizontally (locomotion) and vertically (rearings). Mice were placed in a cage, and their movements were videotaped for analysis. WT and heterozygous mice revealed a similar performance in open field locomotor activity, while AC5−/− showed a small but significant degree of reduction (Fig. 2B and C). To evaluate bradykinesia, pole test was performed. The time until they turned downward (Tturn) and the time until they descended to the floor were measured (Tdesc). We found that AC5−/− had marked deficits in this test; they showed an over 3-fold prolongation of both recording time indexes (Fig. 2D). It was also possible that striatal dysfunction led to choreic or dystonic movements. Such abnormal movements may be demonstrated most readily in mice as a claspings of the limbs that is triggered by tail suspension test (17). However, we found no such abnormal movements in both AC5−/− and WT (data not shown).

AC5 Expression and AC Activity in AC5−/−—These results indicated impairments of striatal functions in AC5−/− presumably induced by the loss of AC5. While AC5 may be striatum-specific with regard to its distribution (18), it remained unknown whether it was dominant for cAMP production in the striatum. AC5 mRNA was expressed at least 10–20-fold more abundantly in the striatum than in the other brain regions, such as the cortex and the cerebellum, in WT (Fig. 3A); this was in agreement with previous findings (18). In AC5−/−, AC5 mRNA expression was negated, but histological examinations revealed no changes such as neuronal loss and/or reactive gliosis at 8–12 weeks old (data not shown). We found, however, that AC activity was greatly decreased in striatal membrane preparations in AC5−/− (Fig. 3B). In contrast, AC activity was significantly, but only to a small degree, decreased in the cortex where AC5 could be detected in WT and showed no difference in the cerebellum where AC5 was scarcely detected in WT. For comparison, AC activity in the heart, another tissue in which AC5 is dominantly expressed (8), was decreased by only 30% (data not shown), indicating that the contribution of AC5 to cAMP production is greater in the striatum than in the heart.

We also examined receptor agonist-stimulated AC activity (Fig. 3C). In general, in many tissues including the heart, marked stimulation of AC is readily attainable with Gα2-coupled receptor agonists, although the inhibition of AC with Gαi-coupled receptor agonists may not always be easy. In the striatum, however, SKF38393, a D1 dopaminergic receptor agonist, modestly stimulated AC activity in WT (40.7 ± 2.6% increase over that with 10 μM GTP, Quinpirole, a D2 agonist, inhibited SKF38393-stimulated AC activity; the inhibition was significant but small (13.5 ± 1.1% decrease). In AC5−/−, the re-
sponses to D1 and D2 dopaminergic receptor agonists were markedly diminished; the D1 dopaminergic agonist-mediated stimulation was very small, and the D2 dopaminergic agonist-mediated inhibition in AC5−/− was hardly detectable. It is tentative to speculate that the loss of D2 agonist-mediated inhibition was due to the loss of AC5, which is Gs-inhibitable, as proposed recently in a similar model (19), while it is also possible that the AC catalytic activity was too low to demonstrate inhibition by AC assays with membrane preparations.

Thus, in vitro AC assays may not be sufficient in terms of sensitivity to study changes in selective dopaminergic signal in AC5−/−. We did not understand, however, why the response to D1 agonist stimulation as shown by percent increase was also attenuated in AC5−/− because other remnant AC isoforms must be able to respond to Gs, if not Gs, stimulation. We also examined cAMP accumulation in intact striatal neuronal cells from the fetus; however, the difference in cAMP production was not as great as in the above AC assays using membrane preparations from adults (data not shown).

Changes in Other Molecules Involved in Dopaminergic Signaling—The disruption of the major striatal AC isoform may change the expression of other molecules involved in dopaminergic signaling. D1 dopaminergic receptor binding sites were modestly decreased in AC5−/−, while D2 receptor binding sites were similar to those in WT (Fig. 4A). The short, but not the long, form of Gs protein expression was decreased in AC5−/− (Fig. 4B) presumably due to the loss of positive feed forward regulatory loop. Western blotting for various molecules, using either the membrane preparation or whole tissue homogenates, revealed that the protein expression of Golf, Gi, Gq, Gβ, and PKA (the α catalytic subunit) were not changed (data not shown). Changes in neurotransmitters, such as dynorphin, substance P, and enkephalin, were examined by RNase protection assays that may be linked to the activity of D1 and D2 dopaminergic receptors. The expression of dynorphin, which acts on presynaptic α-receptors to inhibit AC (20), was modestly increased (Fig. 4C). In contrast, the expressions of enkephalin and substance P were unchanged (Fig. 4C). The expressions of glutamic acid decarboxylase and tyrosine hydroxylase, which are involved in the synthesis of γ-aminobutyric acid and dopamine, respectively, were also unchanged as determined by immunoblotting (data not shown). The above findings suggested that the expression of some molecules, i.e. D1 dopaminergic receptors, Gs, and dynorphin, was changed in such a way to suppress the D1 dopaminergic pathway despite the disruption of the major AC isoform.

We then examined whether there was any increase in the expression of other AC isoforms in AC5−/−. Since AC isoform antibodies that can convincingly determine the level of protein expression are not available, we quantitated the mRNA expression of the AC isoforms by RNase protection assays (Fig. 4D). All AC isoforms except AC4 and AC8 were detected. Among these isoforms, we found a modest increase of AC6, the most relevant isoform to AC5, as well as AC2, AC7, and AC9 but not AC1 and AC3 in AC5−/−. We thought, however, that such small increases in the expression of AC isoforms were not sufficient to explain decreases in the expression of Gs and D1 dopaminergic receptors, which occurred as if to inhibit the D1 dopaminergic pathway.

Dopaminergic Agonists Improved Motor Function in AC5−/−—If either or both D1 and D2 dopaminergic signals were attenuated in AC5−/− leading to motor dysfunction in vivo, then stimulation of dopaminergic receptors, D1 and/or D2, with specific agonists may restore the function. Administration of SKF38393 (25 or 50 mg/kg), a D1 dopaminergic agonist, increased locomotor activity in both WT and AC5−/−. In particular, locomotor activity response appeared pronounced, and might be supersensitive, in AC5−/− relative to WT (Fig. 5A).

This finding was reminiscent of the supersensitive response of the direct pathway neurons observed in dopamine depletion of Parkinson’s disease in which the D1 dopaminergic function becomes supersensitive but is accompanied by an actual reduction of D1 dopamine receptor levels (21, 22) (Fig. 4A). SKF38393 did not improve Rotarod performance in both WT and AC5−/− (Fig. 5B). We then examined the effect of cabergoline (0.2 or 1.0 mg/kg), a D2 dopaminergic agonist that has been used in the treatment of Parkinson’s disease. Cabergoline had no significant effect on locomotor activity in both WT and AC5−/−, although both showed a tendency of small increases (Fig. 5D). In contrast, cabergoline improved Rotarod performance selectively in AC5−/−; their performance reached an equivalent level to that of WT (Fig. 5E), while cabergoline essentially had no effect on WT, suggesting that coordination in AC5−/− was restored by D2 dopaminergic stimulation. We also examined the effect of these agonists on pole test performance (Fig. 5, C and F). Both SKF38393 and cabergoline improved pole test performance in AC5−/−, the latter of which induced a dramatic improvement even with a lower dose (0.2 mg/kg).
DISCUSSION

We have demonstrated that the disruption of the AC5 gene led to a major deficit in AC activity in a striatal specific manner and an abnormal coordination represented by impaired rotarod performance as well as other motor disorders that mimicked Parkinson’s disease. Selective stimulation of D2 dopaminergic receptors by cabergoline restored coordination, suggesting that the attenuation of D2 dopaminergic signal underlined abnormal coordination in AC5−/− and that D2 dopaminergic signal targets AC5 as a major effector isoform. Locomotor activity was also attenuated and restored by selective D1 dopaminergic stimulation, suggesting that this dopaminergic signal also targets AC5. In contrast, both dopaminergic signals may be able to couple to other AC isoforms as well because D1 or D2 dopaminergic stimulation could restore specific motor function, i.e. coordination or locomotion, respectively. Nevertheless such selective dopaminergic agonist stimulation could not restore all of the motor disorders, indicating that AC5 is essential in balancing and maintaining both coordination and locomotion and may provide the site of convergence of both D1 and D2 dopaminergic signals. D1 and D2 are the most abundant dopaminergic receptors expressed in the brain, and both are involved in the two major striatal output pathways, i.e. the “direct” and the “indirect” pathways, which are dominantly mediated by D1 and D2 receptors, respectively. Although it is still unknown whether these receptor subtypes are expressed in the distinct populations of striatal neurons (23) or within the same populations (24), it has been believed that the parallel and balanced activation of these two pathways and their synergistic action control striatal motor functions. Our findings indicate that the parallel and balanced activation is maintained by the presence of AC5 that is coupled to both dopaminergic pathways. In the absence of AC5, AC6 and AC1 are still present but cannot fully compensate for the function of AC5.

The supersensitive response to D1 dopaminergic stimulation (Fig. 5A) mimicked the supersensitivity in Parkinson’s disease. As in Parkinson’s disease, AC5−/− also had decreased D1 dopaminergic receptor expression (21, 22). Because there was no up-regulation of G protein or PKA expression, changes responsible for this supersensitization must be located downstream of PKA, although we do not deny that compensation in the AC pathway could include increased translation and/or post-translational activation of remaining AC isoforms or other pathway components. The exact molecular mechanisms for this paradoxical phenomenon have also remained unexplained in Parkinson’s disease, but a very recent study suggested that a switch in the regulation of downstream mitogen-activated protein kinase signal may be involved (25). The dopamine depletion in Parkinson’s disease and the lack of its major effector enzyme isoform may be similar in many aspects, and thus AC5−/− may be useful to explore the molecular mechanisms for supersensitization in future studies.

The robust nature of our finding, however, suggests that the neurotransmitter signal at the level of an effector isoform, i.e. the integration of multiple receptor subtype signals on one effector isoform, may be as equally important as the neurotransmitter signal at the level of their respective receptor subtypes in regulating neuronal functions that has been more widely recognized. Our findings also imply that targeting an AC isoform(s), such as AC5, in future pharmacotherapy may be an effective way to treat motor dysfunction in human diseases (26).

Acknowledgments—We thank Dr. Kazushige Tsuchara (University of Tokyo) for helpful discussion and Dr. Hideaki Hori (Yokohama City University) for editorial assistance.

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*J. Biol. Chem. 2003, 278:16936-16940.*
doi: 10.1074/jbc.C300075200 originally published online March 28, 2003

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