Balance and Hearing Deficits in Mice with a Null Mutation in the Gene Encoding Plasma Membrane Ca\(^{2+}\)-ATPase Isoform 2*

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Peter J. Kozel†, Rick A. Friedman§, Lawrence C. Erway¶, Ebeenezer N. Yamoah∥, Lynne H. Liu‡, Tara Riddle§, John J. Duffy‡, Thomas Doetschman‡, Marian L. Miller∗∗, Emma Lou Cardell, and Gary E. Shull† ‡‡

From the Departments of Molecular Genetics, Biochemistry and Microbiology, †Cell Biology and Anatomy, and ‡‡Environmental Health, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267, the †Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221, and the †House Ear Institute, Los Angeles, California 90067

Plasma membrane Ca\(^{2+}\)-ATPase isoform 2 (PMCA2) exhibits a highly restricted tissue distribution, suggesting that it serves more specialized physiological functions than some of the other isoforms. A unique role in hearing is indicated by the high levels of PMCA2 expression in cochlear outer hair cells and spiral ganglion cells. To analyze the physiological role of PMCA2 we used gene targeting to produce PMCA2-deficient mice. Breeding of heterozygous mice yielded live homozygous mutant offspring. PMCA2-null mice grow more slowly than heterozygous and wild-type mice and exhibit an unsteady gait and difficulties in maintaining balance. Histological analysis of the cerebellum and inner ear of mutant and wild-type mice revealed that null mutants had slightly increased numbers of Purkinje neurons (in which PMCA2 is highly expressed), a decreased thickness of the molecular layer, an absence of otocinia in the vestibular system, and a range of abnormalities of the organ of Corti. Analysis of auditory evoked brainstem responses revealed that homozygous mutants were deaf and that heterozygous mice had a significant hearing loss. These data demonstrate that PMCA2 is required for both balance and hearing and suggest that it may be a major source of the calcium used in the formation and maintenance of otocinia.

Calmodulin-dependent plasma membrane Ca\(^{2+}\)-ATPases (PMCA)* are highly regulated enzymes that maintain the appropriate concentrations of intracellular free Ca\(^{2+}\) by extruding Ca\(^{2+}\) from the cell (1, 2). There are four mammalian PMCA isoforms (PMCA1–4), each encoded by a distinct gene (3–8), and additional diversity is generated by alternative splicing of exons encoding the regulatory domains (7, 9–11). Variants of PMCA1 and PMCA4 are expressed in many different tissues and cell types, whereas variants of PMCA2 and PMCA3 exhibit a highly restricted distribution (5, 8, 12). This suggests that specific isoforms and splice variants serve different physiological functions. However, despite extensive information about PMCA structural diversity, expression patterns, and biochemical and regulatory characteristics, little is known about the functions of individual isoforms in vivo.

Its unique biochemical characteristics (13, 14) and tissue specificity (5, 10–12) suggest that PMCA2 might serve specialized physiological functions. In situ hybridization studies revealed that expression of PMCA2 is particularly high in Purkinje neurons of the cerebellum (15) and in the spiral ganglion nerves of the inner ear and outer hair cells of the cochlea (16). The observation that PMCA2 is the predominant isoform in outer hair cells (16) suggests that it might be the isoform that is expressed at high levels in stereocilia (17), which comprise hair bundles, the sensory organelles that mediate mechanoelectrical transduction by hair cells of both the vestibular and auditory systems (18, 19). A recent study demonstrated that PMCA activity in the stereocilia of vestibular hair cells regulates hair bundle Ca\(^{2+}\) concentrations and indicated that it might also control the Ca\(^{2+}\) concentrations in the endolymph immediately surrounding the hair bundles (20). The results of our analyses of the PMCA2-deficient mouse, described in the current study, demonstrate that PMCA2 plays critical roles in both the vestibular and auditory systems.

**EXPERIMENTAL PROCEDURES**

**Gene Targeting and Hybridization Analyses**—The targeting construct was prepared using fragments of the mouse Pmca2 gene and the vector, pMJKO, which was described previously (21). A 2.0-kb BspHI fragment beginning in intron 17 and terminating within codon 958 in exon 19 was inserted into a cloning site between the neo gene and the herpes simplex virus thymidine kinase gene in the vector. A 2.3-kb BspHl-NotI fragment beginning within exon 958 and terminating in exon 21 was inserted into another cloning site 5′ to the promoter for the neo gene. Techniques for targeting of ES cells and generation of mutant mice were the same as those used previously (21). Southern blot analysis of ES cell and tail DNAs was performed using a 5′ probe containing parts of exon 17 and intron 17 and a 3′ probe containing parts of intron 20 and exon 21. Northern blot analysis was carried out as described previously (21) using a cDNA probe spanning nucleotides 22–519 of the rat PMCA2 5′-untranslated sequence (3) and total RNA from brains of 6–8-week-old mice.

**Histology and Morphometry**—Cerebella and intact inner ears from 5–6-week-old mice of all three genotypes were removed after perfusion fixation with a phosphate-buffered saline solution containing paraformaldehyde and glutaraldehyde. Temporal bones were decalcified in EDTA for 1 week. The specimens were post-fixed in buffered 1% osmium tetroxide for 1–2 h, dehydrated in a gradient of ethanol washes and propylene oxide, and embedded in Spurr’s resin. Serial 1–2-micron sections perpendicular to the folia of the cerebellum (n = 9 Pmca2+/+, 11 Pmca2+/−, and 8 Pmca2−/− mice) or parallel to the modiolus of the inner ear (n = 8 Pmca2+/+, 8 Pmca2+/−, and 10 Pmca2−/− mice) were cut and stained with toluidine blue.
Panel C

enzyme sites: allele) detected by Southern blot analysis are indicated. Restriction TK herpes simplex virus thymidine kinase gene (Middle, gene.

ing strategy.

Top, negative selection of ES cells.

Bottom, organization of inside and outside probes used for Southern blot analysis. The EcoRI fragments (9.8 kb for wild-type allele; 7 and 4.4 kb for mutant allele) detected by Southern blot analysis are indicated. Restriction enzyme sites: B, BspHII; E, EcoRI. Panel B, Southern blot analysis of EcoRI-digested tail DNAs from offspring of heterozygous matings hybridized with the indicated probes. Panel C, Northern blot analysis of total brain RNA from mice of all three genotypes.

Thickness of the molecular layer, measurements of granule and Purkinje cell numerical density, and granule and Purkinje cell staining quality were obtained using a Zeiss Photomik, a camera lucida, a Jandel Scientific digitizing tablet, and their SigmaScanPro software. At 5–15 sites of optimum orientation for each specimen, the thickness of the molecular layer was measured by digitizing the length of a line drawn perpendicular to and beginning at the layer of Purkinje cell bodies and extending to the surface of the cerebellum. The number of Purkinje cells/100 microns was determined along the plane between the Purkinje cells and granule cells. The number of granule cells/100 microns was determined along a line that was perpendicular to the plane of the Purkinje cell layer, drawn through the granule cell layer, and beginning at each Purkinje cell in each microscopic field. The percentage of granule cells and Purkinje cells that were darkly stained was determined also.

Auditory Brainstem Responses—Mice were anesthetized with avertin, and ABR measurements were performed within a sound-attenuated chamber as described previously (22). Three electrodes, one under each ear and one at the top of the skull, were inserted subcutaneously. The mice were placed between two high frequency transducers, and tubing was oriented to direct sound stimuli into the ear canals. The ABR was computer-averaged (time-locked with onset of 128–1024 stimuli, at 20/s) out of the continuous electroencephalographic activity, and the threshold of hearing was determined by observing the lowest intensity of sound required to elicit a characteristic waveform.

RESULTS

Generation of Mutant Mice—The gene encoding PMCA2 (Atp2b2) was disrupted in ES cells using a targeting construct (Fig. 1A) in which the neo gene was inserted into exon 19, which encodes transmembrane domains essential for transport activity. Chimeric male mice were prepared using these ES cells and bred with wild-type Black Swiss mice. Germline transmission of the mutant allele was achieved, and breeding of Pmca2 Academic mice yielded live offspring of all three genotypes (Fig. 1B) in a 1:2:1 Mendelian ratio (74 Academic, 151 Academic, 87 Academic). Northern blot analysis demonstrated that the mutation caused a reduction of PMCA2 mRNA expression in brain of Pmca2 Academic mice and eliminated expression in Pmca2 Academic mice (Fig. 1C).

Gross Phenotype—Homozygous mutant mice grew to adulthood (with the oldest currently at 8 months of age) with no excess mortality, but they exhibited severe ataxia that was clearly apparent by 12 days of age and had reduced body weight (~20–30% less than Pmca2 Academic and Pmca2 Academic mice). Pmca2 Academic mice did not display these outward manifestations of an abnormal phenotype. As shown in panels A–C of Fig. 2, young Pmca2 Academic mice had great difficulty maintaining their balance when walking or standing and frequently fell onto their sides. The mice often rolled onto their backs and would flail their hind legs and tail while attempting to right themselves. By 5 months of age, the Pmca2 Academic mice improved in their ability to maintain an upright position, and many of them tended to alternately hyperextend (in the manner shown in Fig. 2B) and clench their rear legs and feet together (Fig. 2D). In addition to the apparent balance deficit, Pmca2 Academic mice showed no response to a hand clasp, indicating that their hearing was impaired.

Histological and Morphometric Analyses of Cerebellum—Because PMCA2 is expressed at high levels in Purkinje neurons (15), we evaluated the morphology of the cerebellum. Heterozygous mice were not significantly different from wild-type mice in any of the measured parameters. In null mutants, the numerical density of Purkinje cells was increased (2.4 ± 0.0 and 1.9 ± 0.1 cells/100 microns for Pmca2 Academic and Pmca2 Academic mice, respectively; p < 0.02), and the numerical density of granule cells was reduced (6.4 ± 0.24 and 7.7 ± 0.14 cells/100 microns for Pmca2 Academic and Pmca2 Academic mice, respectively; p < 0.01). Finally, there was no significant difference in the staining intensity of Purkinje cells, but the percentage of granule cells that were densely stained (pyknotic) was increased (3.9 ± 1.5% for Pmca2 Academic and 0.28 ± 0.16% for Pmca2 Academic, p < 0.01). Finally, the molecular layer was thinner in Pmca2 Academic mice (171 ± 10 microns for Pmca2 Academic and 206 ± 11 microns for Pmca2 Academic, p < 0.05).

Histology of the Inner Ear—Light microscopic analysis of sections of the vestibular system from mice of all three genotypes revealed no apparent histopathology of the semicircular canals or cristae ampullares. Hair cells, support cells, and innervation of the sensory epithelium in the utricle and saccule of both wild-type and mutant mice appeared normal, and the otolithic membrane was present above the macula in each chamber. Whereas otoconia were numerous and clearly visible in the vestibular organs of Pmca2 Academic mice (as shown in the saccule, Fig. 3A) and were also observed in the vestibule of Pmca2 Academic mice (data not shown), these calcium carbonate crystals were missing in both the saccule (Fig. 3B) and utricle (data not shown) of Pmca2 Academic mice.

Fig. 1. Targeted disruption of the Pmca2 gene. Panel A, targeting strategy. Top, organization of the relevant region of the wild-type gene. Middle, targeting construct with neo gene disrupting exon 19. The herpes simplex virus thymidine kinase gene (TK) was included for negative selection of ES cells. Bottom, targeted Pmca2 gene and location of inside and outside probes used for Southern blot analysis. The EcoRI fragments (9.8 kb for wild-type allele; 7 and 4.4 kb for mutant allele) detected by Southern blot analysis are indicated. Restriction enzyme sites: B, BspHII; E, EcoRI. Panel B, Southern blot analysis of EcoRI-digested tail DNAs from offspring of heterozygous matings hybridized with the indicated probes. Panel C, Northern blot analysis of total brain RNA from mice of all three genotypes.

Fig. 2. Pmca2 Academic mice exhibit an unsteady gait and difficulty in maintaining balance. A–C, three views of 5-week-old Pmca2 Academic mice showing balance deficit. D, 7-month-old Pmca2 Academic mouse turning its head and flailing its tail while righting itself after a fall. Note the clenched rear legs and feet that are typical of older null mutants.
The objectives of this study were to develop a Pmca2-deficient mouse and to analyze its phenotype to better understand the physiological functions of this isoform in vivo. The absence of detectable PMCA2 mRNA in homozygous mutants confirmed that our targeting strategy had produced a null mutation. Homozygous mutants were born in the normal Mendelian ratio and exhibited no excess mortality, demonstrating that PMCA2 is not essential for embryonic development or survival after birth. Severe ataxia, however, was clearly apparent before 2 weeks of age, and additional studies revealed defects of both the vestibular and auditory systems.

Because in situ hybridization studies have shown that PMCA2 is expressed at high levels in Purkinje neurons (15), we considered the possibility that the ataxia was because of a cerebellar defect. Morphological alterations in the Pmca2−/− cerebellum included a decrease in the number of granule cells, a slight increase in the number of Purkinje cells, and a decreased thickness of the molecular layer, which suggests the
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possibility of decreased arborizations of the Purkinje cell dendritic processes. Although it is conceivable that these changes contribute to the phenotype, they seem minor compared with those seen in the pcd (Purkinje cell degeneration) mutant mouse, which loses most of its Purkinje cells yet shows only moderate ataxia (23). This led us to consider the possibility of defects in the vestibular system, which senses equilibrium and balance (18).

The vestibular system consists of the semicircular canals, which detect changes in angular acceleration, and the utricle and saccule, which detect changes in linear acceleration and head position with respect to gravity. Histological abnormalities were not observed in the semicircular canals of Pmca2−/− mice, and the crista ampullaris, with its sensory hair cells and gelatinous cupula within the ampulla of each duct, appeared normal. Similarly, we observed no abnormalities of the maculae of the saccule and utricle, and in each chamber the gelatino nous otolithic membrane that overlies the neuroepithelium was intact. Pmca2-null mutants, however, lacked otoconia in both the saccule and utricle. Because these calcium carbonate crystals are very dense relative to the endolymph and otolithic membrane, their mass is readily shifted in response to gravity or linear acceleration, thereby shifting the otolithic membrane and stimulating the hair cells. The fact that null mutants exhibited difficulties in maintaining their balance even when standing still, which would not involve the semicircular canals, is consistent with a deficit in the saccule and utricle. Thus, the lack of otoconia provides a striking histopathological correlate of the defect in balance.

Recent evidence indicates that otoconia grow by accretion of calcium carbonate prior to and shortly after birth (24), although the mechanism by which they are formed and the source of the calcium is not known. There is no information about the expression of PMCA2 in the vestibular system, but a recent study has shown that it is expressed in multiple cell types bathed by the endolymph of the cochlear duct (16), which is continuous with the membranous labyrinth of the vestibular system. The absence of otoconia in Pmca2−/− mice raises the interesting possibility that these calcium carbonate crystals are formed and/or maintained, at least in part, by Ca2+ extruded into the endolymph by PMCA2.

The most striking observations were the hearing deficits and histopathology of the organ of Corti in both Pmca2−/− and Pmca2+/− mice. ABR measurements demonstrated that Pmca2−/− mice are profoundly deaf and that Pmca2+/− mice exhibit a severe hearing loss. The hearing loss in heterozygous mutants is of considerable interest because it shows that a null mutation in the Pmca2 gene can cause an autosomal dominant hearing loss. The observed histopathology is consistent with the possibility that PMCA2 plays a role in the development of the organ of Corti and spiral ganglion neurons; however, there were regions in which these structures appeared relatively normal. Thus, an alternative possibility is that the organ of Corti and spiral ganglion neurons develop normally and then degenerate with age, as seen in the deafness mouse (25). The expression of PMCA2 in outer hair cells (16) suggests that it is the isofrom that is present at high levels in the hair bundle (17, 26) and might therefore play an important role in sensory transduction. PMCA activity in the hair bundle has been shown to regulate Ca2+ concentrations within the stereocilia and may also generate a Ca2+ gradient near the stereocilia that might influence the sensitivity of the hair cell (20). Additional studies will be needed to determine the time course and mechanisms underlying the histopathology of the organ of Corti and whether PMCA2 plays a direct role in mechanoelectrical transduction by hair cells.

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