Null Mutation in the Gene Encoding Plasma Membrane Ca\(^{2+}\)-ATPase Isoform 2 Impairs Calcium Transport into Milk*

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The means by which calcium is transported into the milk produced by mammary glands is a poorly understood process. One hypothesis is that it occurs during exocytosis of secretory products via the Golgi pathway, consistent with the observation that the SPCA1 Ca\(^{2+}\)-ATPase, which is expressed in the Golgi, is induced in lactating mammary tissue. However, massive up-regulation of the PMCA2bw plasma membrane Ca\(^{2+}\)-ATPase also occurs during lactation and is more strongly correlated with increases in milk calcium, suggesting that calcium may be secreted directly via this pump. To examine the physiological role of PMCA2bw in lactation we compared lactating PMCA2-null mice to heterozygous and wild-type mice. Relative expression levels of individual milk proteins were unaffected by genotype. However, milk from PMCA2-null mice had 60% less calcium than milk from heterozygous and wild-type mice, the total milk protein concentration was lower, and an indirect measure of milk production (litter weights) suggested that the PMCA2-null mice produce significantly less milk. In contrast, lactose was higher in milk from PMCA2-null mice during early lactation, but by day 12 of lactation there were no differences in milk lactose between the three genotypes. These data demonstrate that the activity of PMCA2bw is required for secretion of much of the calcium in milk. This major secretory function represents a novel biological role for the plasma membrane Ca\(^{2+}\)-ATPases, which are generally regarded as premier regulators of intracellular Ca\(^{2+}\).

The mammary gland transports large amounts of Ca\(^{2+}\) from the blood to the milk via the mammary secretory cells. These transcellular Ca\(^{2+}\) fluxes must be rigorously controlled to prevent cytotoxicity (1). This is a formidable task, because mammary glands store 12–30 \(\mu\)mol of Ca\(^{2+}\)/g of tissue compared with less than 1 \(\mu\)mol/g in non-mammary tissues (2, 3). The plasma membrane Ca\(^{2+}\)-ATPase (PMCA), secretory pathway Ca\(^{2+}\)-ATPase (SPCA), and sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) are up-regulated in lactating mammalian glands (4–6). However, PMCA2bw is the most abundant Ca\(^{2+}\)-ATPase expressed during lactation, and with an ~100-fold up-regulation of PMCA2 protein during lactation its expression levels are closely correlated with milk production and calcium secretion (4, 5).

PMCA proteins, along with SERCA and SPCA pumps, are responsible for establishing and maintaining appropriate intracellular Ca\(^{2+}\) levels (7–11) with SERCAs and SPACs sequestering Ca\(^{2+}\) intracellular organelles and PMCA providing a high affinity system for extrusion of Ca\(^{2+}\). As far as we are aware, the alternative hypothesis, that Ca\(^{2+}\) is secreted directly via the calmodulin-sensitive plasma membrane Ca\(^{2+}\)-pump, has not been considered previously. In the current study, we show that mice homozygous for the loss of PMCA2 isoforms particularly with respect to phosphorylation and calmodulin stimulation (12–14) with splice variant b of PMCA2 exhibiting a very high affinity for calmodulin (15), thereby making it particularly effective for Ca\(^{2+}\) extrusion. Alternative splicing at site C (COOH-terminal tail) has been shown to alter the regulatory properties of PMCA isoforms particularly with respect to phosphorylation and calmodulin stimulation (12–14) with splice variant b of PMCA2 exhibiting a very high affinity for calmodulin (15), thereby making it particularly effective for Ca\(^{2+}\) extrusion. Alternative splicing at site C (COOH-terminal tail) has been shown to alter the regulatory properties of PMCA isoforms particularly with respect to phosphorylation and calmodulin stimulation (12–14) with splice variant b of PMCA2 exhibiting a very high affinity for calmodulin (15), thereby making it particularly effective for Ca\(^{2+}\) extrusion. Alternative splicing at site C (COOH-terminal tail) has been shown to alter the regulatory properties of PMCA isoforms particularly with respect to phosphorylation and calmodulin stimulation (12–14) with splice variant b of PMCA2 exhibiting a very high affinity for calmodulin (15), thereby making it particularly effective for Ca\(^{2+}\) extrusion. Alternative splicing at site C (COOH-terminal tail) has been shown to alter the regulatory properties of PMCA isoforms particularly with respect to phosphorylation and calmodulin stimulation (12–14) with splice variant b of PMCA2 exhibiting a very high affinity for calmodulin (15), thereby making it particularly effective for Ca\(^{2+}\) extrusion.
cally the PMCA2bw splice variant (the characteristics of which should allow the enzyme to function as a high efficiency Ca\(^{2+}\) extrusion system on the apical membrane), is necessary to produce the high levels of Ca\(^{2+}\) in milk.

**EXPERIMENTAL PROCEDURES**

**Animal Procedures**—All animal procedures were approved by the National Animal Disease Center’s Animal Care and Use Committee. PMCA2 knock-out mice were prepared as described (20). Mice were housed individually in hanging basket cages with sawdust bedding. The mice were genotyped using the following primers in a single reaction.

![Brain PMCA2](image1)

**Mammary PMCA2**

A. PMCA2 in brain microsomes from all three genotypes on day 12 of lactation. B–D, PMCA2, SPCA1, and SERCA2, respectively, in mammary gland microsomes from all three genotypes on day 12 of lactation. Expression levels for each protein in the bar graphs are normalized to those of Pmc2\(^{+/-}\) mice. Data are means ± S.E. (n = 4–5 mice/data point).

**Gel Electrophoresis and Western Blotting**—The methods used were basically as described previously (29). Briefly, microsomes were incubated for 15 min at room temperature in a modified Laemmli buffer containing 150 mM Tris, 2% SDS, 5% 2-ME, and 65 mM dithiothreitol. Samples were then electrophoresed for 1.5 h at 125 volts in a 6% Tris-glycine gel (Novex, San Diego, CA). Proteins were transferred to nitrocellulose membranes for 1 h at 25 volts in 0.7 M glycine, 0.025 M Tris at pH 7.4.

**RESULTS**

**Ca\(^{2+}\)-ATPase Expression in Brain and Lactating Mammary Tissue**—Western blots were performed on brain and lactating mammary tissue collected from mice of all three genotypes on day 12 of lactation. Fig. 1A shows the expected decline (58 ± 8%) in brain PMCA2 protein expression in Pmc2\(^{+/-}\) mice compared with Pmc2\(^{+/-}\) mice, whereas Pmc2\(^{-/-}\) mice ex-
press no PMCA2. In contrast, PMCA2 expression in lactating mammary tissue declined only 38 ± 9% (p < 0.05) in the Pmca2–/– mice compared with Pmca2+/+ mice (Fig. 1B). SPCA1 in lactating mammary tissue was up-regulated 22 ± 10% and 42 ± 9% (p < 0.01) in Pmca2+/+ and mice Pmca2–/–, respectively (Fig. 1C), whereas SERCA2 expression increased 48 ± 11% (p < 0.01) and 98 ± 12% (p < 0.01) in Pmca2+/+ and Pmca2–/– mice, respectively (Fig. 1D).

Milk Calcium Is Sharply Reduced by Loss of PMCA2—Milk calcium was measured in mice of all three genotypes on days 6–12 of lactation. Milk calcium concentrations in both Pmca2+/+ and Pmca2–/– mice were 95 mM, whereas milk calcium in Pmca2+/– mice was reduced 60% to 38 mM (p < 0.01) during the sampling period (Fig. 2A). Because the bulk of calcium secreted into milk is thought to arrive as a protein complex, the data for calcium were also corrected for milk protein secretion (Fig. 2B). By either measure, Pmca2+/– mice secreted significantly less calcium into their milk than Pmca2+/+ or Pmca2–/– mice.

Effect of Mouse Genotype on Milk Protein and Lactose—Analysis of whey acid protein and general milk proteins (Fig. 3A) showed that the mouse genotype had no effect on the overall patterns of milk protein expression. Compared with Pmca2+/+ mice, total milk protein concentration was reduced 39% (p < 0.01) in Pmca2–/– mice during early lactation but only 14% (p < 0.05) by day 12 (Fig. 3B). Milk protein concentrations in Pmca2–/– mice were more variable and tended to be reduced compared with those of Pmca2+/+ mice, and milk proteins were the same for both Pmca2+/+ or Pmca2–/– mice by day 12 of lactation.

In contrast, Pmca2–/– mice secreted more lactose than either Pmca2+/+ or Pmca2+/+ mice on days 6 (p < 0.01) and 8 (p < 0.05) of lactation (Fig. 4A). By day 10 of lactation all three genotypes had equal lactose concentrations in their milk.

Effect of Mouse Genotype on Milk Production—Because milk production by nursing mice cannot be measured directly, an indirect measurement of relative milk production by mice of all three genotypes was obtained by weighing their litters on days 2–12. Litter sizes were the same for all genotypes. Litters from Pmca2–/– mice were significantly lighter by day 4 of lactation (Fig. 4B). The growth of Pmca2–/– litters lagged behind those of Pmca2+/+ or Pmca2+/+ litters throughout the sampling period.

General Calcium Homeostasis of the Genotypes—Table I shows blood calcium and 1,25(OH)2D3 for the three genotypes on day 12 of lactation. All mice had normal blood values. The blood calcium of Pmca2+/+ or Pmca2+/+ mice was lower (p < 0.05) and their blood 1,25(OH)2D3 was higher (p < 0.05) than that of Pmca2–/– mice.

**DISCUSSION**

There is considerable information about the structures, tissue distributions, and biochemical characteristics of PMCA isoforms. Only recently, however, with the development of gene-targeted animals, has it been possible to begin systematic studies of the in vivo physiological functions of individual isoforms and their splice variants (8, 10–12, 19–21, 30, 31). Analysis of the lactating mammary gland as a site of enormous calcium transport and storage offers the opportunity to gain insights regarding the specific physiological function of PMCA2bw, the most highly expressed Ca2+-ATPase in lactating mammary tissue (4, 5). Therefore the objective of this study was to determine the physiological role of PMCA2bw in calcium transport into milk and its general effects on milk composition.

Our data show that PMCA2bw is the primary controller of milk calcium concentration as the absence of PMCA2bw (Fig. 1B) results in a ~60% decline in milk calcium concentration (Fig. 2A). Interestingly, a 40% decline in PMCA2bw expression...
in Pmca2°/° mice compared with Pmca2°/+ mice had no effect on milk calcium concentrations (Fig. 2A), suggesting either that apical PMCA2 activity of wild-type mice is not rate-limiting for calcium secretion or that the small increases in the expression of SPCA1 and SERCA2b seen in Pmca2°/+ mice (Fig. 1, C and D) provide some compensation for the reduction in PMCA2 levels. However, much larger increases in the expression of SPCA1 and SERCA2b seen in Pmca2°/+ mice (Fig. 1, C and D) were unable to fully compensate for the complete loss of PMCA2bw in Pmca2°/° mice with respect to milk calcium (Fig. 2, A and B). If the increased expression of SPCA1 and SERCA2b in Pmca2°/+ mammary glands does, in fact, provide partial compensation for the deficit in milk calcium, then it would imply that these intracellular pumps play a subsidiary role in the delivery of calcium to the milk, most likely via exocytosis. However, if such compensation is occurring in the knock-out mice, then the normal contribution of PMCA2 to the calcium composition of the milk is likely to be even greater than indicated by the −60% reduction observed in the knock-out mice. Thus, much of the calcium in milk appears to be secreted directly across the apical membrane via PMCA2bw.

Intra-organelle calcium plays a critical role milk protein synthesis, casein phosphorylation, and to a lesser extent protein secretion (32, 33). Endoplasmic reticulum calcium is required for milk protein synthesis, as depletion of calcium from the endoplasmic reticulum inhibits milk protein synthesis (33, 34). The Golgi on the other hand contains the bulk of lactating mammary tissue calcium (35), and this calcium pool is required for normal casein phosphorylation as well as casein micelle formation (33, 34). Despite the many roles for calcium in milk protein synthesis and processing, we found no effect of genotype on either the phosphorylation of milk proteins (data not shown) or the expression of individual milk proteins (Fig. 3A).

However, total milk protein concentration was significantly reduced in Pmca2°/+ mice (Fig. 3B) and to a lesser extent in Pmca2°/° mice. This result suggests that PMCA2bw may affect intra-organelle calcium pools as has been seen for other PMCA pumps (36). Therefore, the loss of PMCA2bw may contribute to the reduction of total milk protein seen in Pmca2°/+ mice. However, milk lactose is the primary osmotic regulator in milk. Thus, increased lactose concentrations are associated with higher milk water content (37). The largest decrease in total milk protein (39%) in Pmca2°/+ mice occurs on day 6 of lactation when Pmca2°/+ mice have significantly higher milk lactose concentrations (Fig. 4A). The increased water transport associated with these higher milk lactose concentrations would lead to the observed decreased milk protein concentrations because of simple dilution. Once lactose concentrations are equal (days 10–12) for all three genotypes, the reduction in milk protein in Pmca2°/+ and Pmca2°/° mice is only 14% compared with Pmca2°/+ mice, whereas milk calcium and milk calcium/mg of milk protein has reached its nadir at a 60% reduction in Pmca2°/+ mice.

Studies by others have suggested that all calcium (free and bound) arrives in milk via the secretory pathway (22–26) with little if any milk calcium arriving in milk via direct extrusion across the apical membrane. However, the data supporting these conclusions are indirect at best. The effects of the loss of PMCA2bw on milk calcium suggest that the primary function of PMCA2bw is apical transport of calcium into milk as PMCA2bw is concentrated on the apical membrane of lactating mammary tissue (5). It should be pointed out that PMCA2bw protein turns over rapidly in lactating tissue as the apical membrane is secreted into the milk as a part of milk fat secretion (5). This requires new synthesis of PMCA2bw to meet the needs of apical membrane renewal (5). Therefore, there is always newly synthesized PMCA2bw in transit to the apical membrane of lactating cells. The work of Taylor et al. (38) suggests that PMCA2bw in transit to the plasma membrane of a cell may contribute to Golgi calcium accumulation. By Western blotting their data showed some PMCA transiting the Golgi. However, their Golgi calcium transport studies did not address the role of the Golgi resident and thapsigargin-insensitive SPCA in liver Golgi. They concluded that thapsigargin-independent calcium uptake by rat liver Golgi was caused by PMCA. It is more likely that this activity is caused by SPCA. Because SPCA1 is the second most abundant Ca²⁺-ATPase in lactating tissue, it is likely the primary controller of calcium uptake in mammary Golgi (5). In the case of lactating mammary tissue, any potential contribution of PMCA2bw to intra-organelle calcium accumulation will only be known following direct measures of the relative contributions of each pump to Golgi and endoplasmic reticulum calcium uptake. Finally, the dogma that little or no calcium enters milk via apical membrane calcium transport needs to be reexamined. The large concentration of PMCA2bw present on the apical membrane along with data showing that the loss of PMCA2bw results in a massive reduction in milk calcium strongly argues for a major calcium transport pathway in the apical membrane of lactating mammary tissue.

Milk production (estimated indirectly by litter weight) is sig-

**TABLE I**

| Genotype | Plasma calcium | Plasma 1,25(OH)₂D³ |
|----------|----------------|-------------------|
| Pmca2°/+ | 8.5 ± 0.3      | 173 ± 15          |
| Pmca2°/− | 8.8 ± 0.4      | 157 ± 18          |
| Pmca2°/− | 9.5 ± 0.3      | 86 ± 14           |

**Fig. 4.** Milk lactose and milk production for Pmca2°/+ and Pmca2°/− mice. A, lactose concentrations in milk from Pmca2°/+ (○), Pmca2°/− (■), and Pmca2°/− (▲) mice on days 6–12 of lactation. B, milk production (estimated from litter weight) in Pmca2°/+ (○), Pmca2°/− (■), and Pmca2°/− (▲) mice on days 6–12 of lactation. Data are means ± S.E. (n = 4–6 mice/data point).
significantly reduced in Pmca2\(^{-/-}\) mice compared with either Pmca2\(^{+/+}\) or Pmca2\(^{+/-}\) mice (Fig. 4B). This may be caused by the reduced calcium available to support lactation in Pmca2\(^{-/-}\) mice but may also be caused by behavioral changes that result from the loss of PMCA2. These animals are deaf and have mobility problems because of loss of balance control that occurs with this mutation. Subjective observations of Pmca2\(^{-/-}\) mothers lead to the conclusion that they had reduced mothering skills. Pups separated from these mothers were not gathered up quickly for suckling as was the case for the other two genotypes. Some of this behavior could be reasonably attributed to their inability to hear their pups and/or the extra effort they had to put into feeding themselves as a result of their mobility problems. There was no abnormal alteration in general calcium metabolism in the Pmca2\(^{-/-}\) that could have contributed to their lower milk production (Table I.) They had higher blood calcium values and lower blood 1,25(OH)\(_2\)D\(_3\) values than either Pmca2\(^{+/+}\) or Pmca2\(^{+/-}\) mice. But these values are in the normal range and just reflect the lower calcium requirements for lactation experienced by the Pmca2\(^{-/-}\) mothers.

In summary, these studies of lactation in Pmca2\(^{-/-}\) mice clearly show that the loss of the major Ca\(^{2+}\)-ATPase expressed in lactating mammary tissue results in a significant reduction in milk calcium and to a lesser extent milk protein. It is concluded that PMCA2bw is the major regulator of calcium required for normal milk production by the mammary gland. The function of PMCA2bw in mammary glands is clearly not to provide fine regulation of cytosolic calcium levels, the role we normally assign to PMCA s. This major macrocalcium secretory function in support of lactation represents a novel biological role for the plasma membrane Ca\(^{2+}\)-ATPases, which are generally regarded as premier regulators of intracellular Ca\(^{2+}\).

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