Localization of plasma membrane and secretory calcium pumps in the mammary gland

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ABSTRACT

Until recently the mechanism for the enrichment of milk with calcium was thought to be almost entirely via the secretory pathway. However, recent studies suggest that a plasma membrane calcium ATPase, PMCA2, is the primary mechanism for calcium transport into milk, highlighting a major role for apical calcium transport. We compared the expression of the recently identified secretory calcium ATPase, SPCA2, and SPCA1, in the mouse mammary gland during development. SPCA2 levels increased over 35-fold during lactation with expression localized to luminal secretory cells, while SPCA1 increased only a modest 2-fold and was expressed throughout the cells of the mammary gland. We also observed major differences in the localization of PMCA2 and PMCA1. Our studies highlight the likely specific roles of PMCA2 and SPCA2 in lactation and indicate that calcium transport into milk is a complex interplay between apical and secretory pathways.

Intracellular free Ca^{2+} levels are under tight regulation and control, with resting free cytosolic Ca^{2+} levels maintained at around 100 nM [1]. This regulation is achieved by proteins responsible for the translocation of Ca^{2+} ions, which include Ca^{2+} channels, Ca^{2+} exchangers, and Ca^{2+} pumps [1,2]. During lactation, the unidirectional transport of Ca^{2+} from the maternal blood supply to the milk must be achieved without causing major disturbances to cellular signaling within the epithelial cells of the mammary gland [3]. Concurrent with tightly maintaining intracellular Ca^{2+} levels, a large gradient between free and total Ca^{2+} between the maternal blood supply and milk is established. Indeed the total Ca^{2+} content of blood is 3 mM [3], while that of the milk is approximately 8 mM in humans, and over 60 mM in other species such as mice [4].

Active transporters of Ca^{2+} ions include the plasma membrane Ca^{2+} ATPases (PMCAs), the sarcoplasmic endoplasmic Ca^{2+}-ATPases (SERCAs). and the secretory pathway Ca^{2+}-ATPases (SPCAs) [2,3]. Consistent with a minor role for the endoplasmic reticulum in Ca^{2+} enrichment of milk, levels of SERCA isoform transcripts expressed in the rat mammary gland (SERCA2 and SERCA3) do not significantly increase during pregnancy or during the early stages

of lactation [5]. In contrast, PMCA2, which has a highly restricted tissue distribution [6,7], increases approximately 30-fold during the early stages of lactation in the rat mammary gland as compared to the almost undetectable levels in nulliparous rats [5,8]. The presence of PMCA2 protein in milk fat globule membranes suggests a predominately apical distribution for PMCA2 during lactation, and a major role in the efflux of Ca^{2+} across the apical membrane into milk [4,8]. The reduction in the Ca^{2+} content of milk in PMCA2 null mice by 60% [9], when combined with only a comparatively modest increase (6–8-fold) in the expression of the secretory pathway pump SPCA1 [5,8], challenges the widely cited view of little to no involvement of direct apical transport of Ca^{2+} and the major role of the Golgi apparatus during lactation [4]. Although a possible role for PMCA2-mediated Ca^{2+} accumulation into the Golgi during trafficking to the membrane has been discussed [9], the recent report of PMCA2 localization to the apical membrane in mammary tissue from a lactating mouse [10] represents further evidence for its direct role in apical calcium transport.

The regulation of key Golgi enzymes involved in post-translational modification of milk proteins and lactose production by Ca^{2+} and Mn^{2+} [3,11], both substrates for the secretory pathway Ca^{2+} pump [12], still suggests a major role for Golgi Ca^{2+} accumulation in lactation, as does the secretion of Ca^{2+} in casein micelles

derived from the Golgi [4]. In this study we sought to address the dichotomy arising from the modest increase in SPCA1 expression and the pronounced increase in PMCA2 during lactation, and the seemingly major role for the secretory pathway in Ca^{2+} secretion for the enrichment of milk with Ca^{2+} [4].

Subsequent to the studies discussed above regarding the expression of SPCA1 and PMCA2 in lactation, a second secretory pathway Ca^{2+}/Mn^{2+} pump isoform has been identified, SPCA2 [13]. Similar to PMCA2, SPCA2 has a more restricted tissue distribution compared to SPCA1. SPCA2 is expressed in the brain and testis [13] as well as the cells of gastrointestinal and respiratory tract and mammary, salivary, and thyroid glands [14]. However, the possible physiological processes where SPCA2 may play a major role are still unclear. In these studies, we addressed the hypothesis that SPCA2 expression undergoes greater changes in expression than SPCA1 during pregnancy and lactation, and that PMCA isoforms and SPCAs have unique cellular distributions during lactation consistent with specific roles in Ca^{2+} homeostasis in the mammary gland and the transport of Ca^{2+} into milk.

Materials and methods

Animals. CBA × C57Bl6 mice were fed standard rodent chow and water ad libitum. Nulliparous animals were euthanized at 14 weeks of age. For all other developmental stages, dams were mated when 12 weeks old. Animals were then sacrificed at the following stages of mammary gland development: mid-pregnancy (~day 10); lactation (day 1); and involution (48 h post-forced weaning).

RNA isolation and real-time RT-PCR. Total RNA was isolated from whole mammary glands using the Qiagen RNeasy mini Kit incorporating an on-column DNase treatment (Qiagen, Doncaster, Vic., Australia). SPCA1 and SPCA2 mRNA levels were quantified using real-time RT-PCR. The mRNA was reverse transcribed using the Omniscript® RT kit (Qiagen) while the real-time PCR step was performed using a TaqMan® universal PCR master mix (Applied Biosystems, Scoresby, Vic., Australia) and the following TaqMan® gene expression assays (Applied Biosystems): Mm00723486_m1 (SPCA1); and Mm01242899_m1 (SPCA2). Expression was normalized to endogenous 18S rRNA levels and is expressed relative to nulliparous animals. Reactions were cycled in an ABI PRISM 7500 Sequence Detector (Applied Biosystems) under the following conditions: 95 °C for 10 min; followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Threshold cycle (C_t) values were used for all analysis. Three separate PCR reactions were performed from each RNA sample from each mouse, to assess variability in C_t responses between replicates and between mice. The mean differences between C_t responses for the different targets, and their standard errors, were estimated using a random effects model, with an additional mouse effect, allowing for the variation in C_t responses between and within mice. Standard errors of further quantities derived from these estimates were determined using the delta technique. Significance was assessed using a z-test based on a normal distribution.

Immunofluorescence (in vivo samples). Glands were sectioned at 5 μM thickness. Sections were fixed with 4% paraformaldehyde in phosphate buffered-saline (PBS). Slides were incubated with primary antibody for 1 h at room temperature. Primary antibodies used included: anti-PMCA1 (1:100, PA1-914; Affinity BioReagents, Golden, CO); anti-PMCA2 (1:800, PA1-915; Affinity BioReagents); affinity purified anti-SPCA1 (1:500); affinity purified anti-SPCA2 (1:100 [13]); and anti-smooth muscle actin (SMA) Cy3 Conjugate (1:400, 1A4; Sigma–Aldrich, Castle Hill, NSW, Australia). Sections were incubated with the secondary antibody Alexa-Fluor®-488 goat anti-rabbit (1:500; Molecular Probes, Mt. Waverley, Vic., Australia) for 40 min, at room temperature. Cells were co-stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich). Fluorescence images were captured with a Nikon Eclipse TE300 microscope (Nikon UK, Kingston, UK), equipped with a 40× oil immersion NA 1.3 objective lens (Nikon), and with MetaFluor® imaging software (Universal Imaging, Downington, PA). All images were analyzed with MetaMorph®, version 6.2 r5 (Universal Imaging).

Cell culture. SCp2 mouse mammary epithelial cells [15] were routinely cultured in Dulbecco's modified Eagle's medium (DMEM)/Hams F-12 as previously described [16]. SCp2 were grown also as a three-dimensional (3D) culture on top of a layer of reconstituted basement membrane gel from Engelbreth–Holm–Swarm extracellular matrix extract, growth factor-reduced (Matrigel; BD Biosciences, Bedford, MA) as previously described [17].

Immunofluorescence (SCp2 cells). Cells were grown in a monolayer (2D) to approximately 90% confluence or in 3D were fixed in an ice-cold methanol/acetone solution (1/1; v/v). Primary antibody dilutions were anti-PMCA1 (1:100), anti-PMCA2 (1:300) and anti-α6 integrin (1:100; MAB 1378; Chemicon International, Temecula, CA, USA). Slides were incubated with secondary antibodies: goat anti-rat Alexa-Fluor® 568 and goat anti-rabbit Alexa-Fluor® 488 (all 1:500; Molecular Probes). Cells were co-stained with DAPI. Confocal analysis was performed using

a Solamere Technology Group (Salt Lake City, UT) spinning disk confocal system. Optical z-sections were acquired at 0.5 μm intervals and are an average of four exposures. All images were analyzed with MetaMorph®, version 6.2 r5.

Immunoblotting. Total cell lysates were isolated from SCp2 cells grown in 2D monolayers (in the presence of normal media or differentiation-inducing media) to reach approximately 90% confluence. For Western blotting antibodies used were anti-β-actin (1:1000, AC-15; Sigma–Aldrich), anti-β-casein (1:1000) prepared from a hybridoma kindly provided by Dr. Kaetzel, Institute of Pathology, Case Western Reserve University, Cleveland, OH [18], goat anti-mouse or goat anti-rabbit IgG horseradish peroxidase-conjugated (both 1:1000; Pierce, Rockford, IL).

Results

To ascertain which isoform, SPCA1 or SPCA2, is the predominant isoform up-regulated during lactation, mRNA levels were compared in mammary glands isolated from nulliparous, pregnant, lactating and involuting mice. Consistent with the increase seen in SPCA1 in rats [5,8], mouse SPCA1 levels increased modestly during pregnancy (~1.5-fold at day 10) and lactation (~2-fold at day 1) (Fig. 1). In contrast, a dramatic up-regulation of SPCA2 was observed in mouse mammary glands during lactation (~35-fold at day 1) with an increase also seen during pregnancy (~8-fold at day 10) compared to nulliparous animals (Fig. 1).

Consistent with its more restricted tissue distribution [14], SPCA2 was expressed predominantly in luminal epithelial cells of the mouse mammary gland during lactation (Fig. 2A), with no expression in cells outside the acini (arrows). SPCA1 staining (Fig. 2B) was seen in all cell types of the tissue section, including myoepithelial and stromal cells (arrows).

Analogous to SPCA1 and SPCA2, PMCA1, and PMCA2 exhibited differential isoform expression in the different cell types of the mammary gland during lactation, with distinct differences in sub-cellular distribution. Fig. 2C shows PMCA2 in the lactating mouse mammary gland. Consistent with its restricted distribution, PMCA2 expression appeared confined to the alveolus, whereas non-acinar cells did not express PMCA2 (arrows). PMCA2 also exhibited an apical localization in many of the acinar structures. Such a distribution suggests that PMCA2 may have a role in the direct translocation of Ca^{2+} across the apical membrane and is consistent with the recent work of VanHouten et al. [10]. In sharp contrast to PMCA2, PMCA1 appeared to be ubiquitously expressed
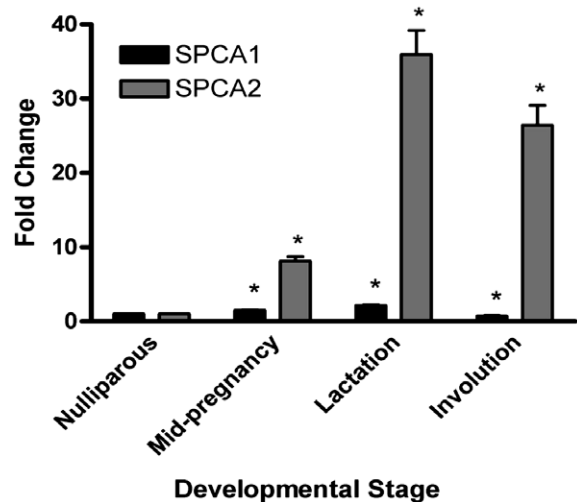

Fig. 1. SPCA2 mRNA levels increase more than SPCA1 during murine mammary gland development. SPCA2 and SPCA1 mRNA levels during murine mammary gland development (relative to nulliparous). Bars represent means ± SEM ($n = 3–4$ mice) and are representative of two independent real-time RT-PCRs. The asterisk (*) denotes a significant difference ($p < 0.05$) compared to nulliparous gland, using a z-test based on a normal distribution.
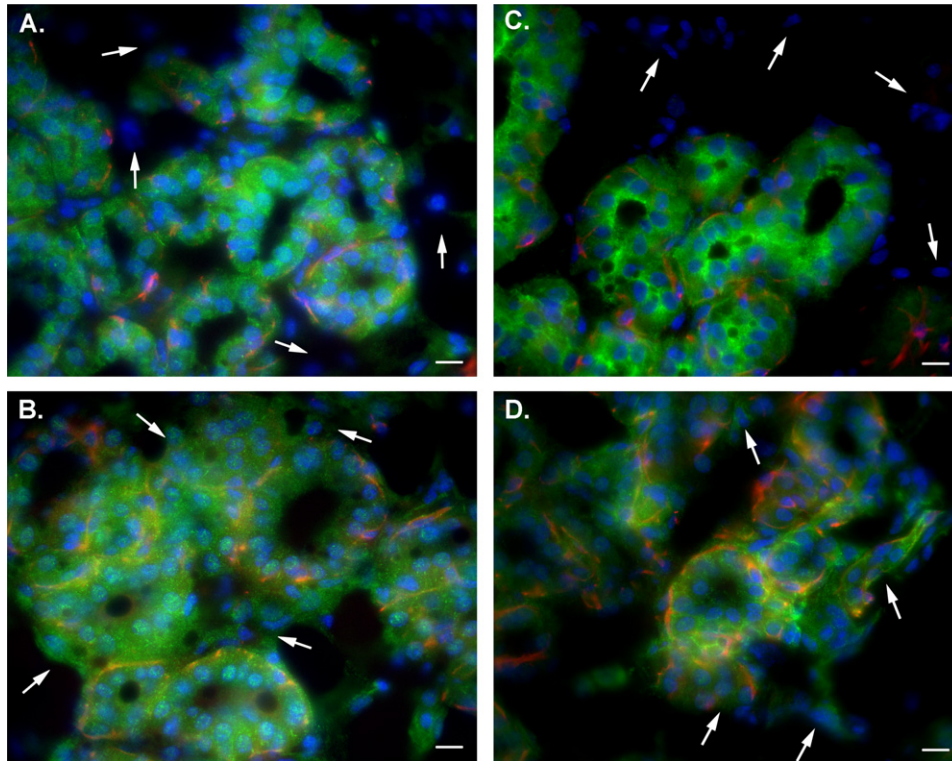
Fig. 2. Immunolocalization of calcium ATPases in the lactating mouse mammary gland. Mammary gland sections isolated from lactating mice and immunostained for SPCA2 (A), SPCA1 (B), PMCA2 (C) or PMCA1 (D). All ATPases are shown in green with SMA (red) overlaid with the DAPI (blue) stain. (A) SPCA2. White arrows show cells which do not express SPCA2 outside the acini. (B) SPCA1. White arrows show cells not part of secretory acini that express SPCA1. (C) PMCA2. White arrows show cells which do not express PMCA2 outside the acini. (D) PMCA1. White arrows show cells not part of secretory acini that express PMCA1. Scale bars represent 10 μm in all panels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

in the mammary gland sections (Fig. 2D) with expression outside the acini (arrows). PMCA1 had a more basolateral distribution, consistent with a possible role in regulation of cytosolic free Ca^{2+} in cells of the mammary gland, rather than the active transport of Ca^{2+} into milk.

To confirm the distinct differences between PMCA1 and PMCA2 in sub-cellular distribution the mouse mammary SCp2 cell line was differentiated to produce 3D acini. These cells polarize and form a lumen and secrete the milk protein, β-casein (supplementary Fig. 1), analogous to primary cultures [19]. PMCA1 and PMCA2
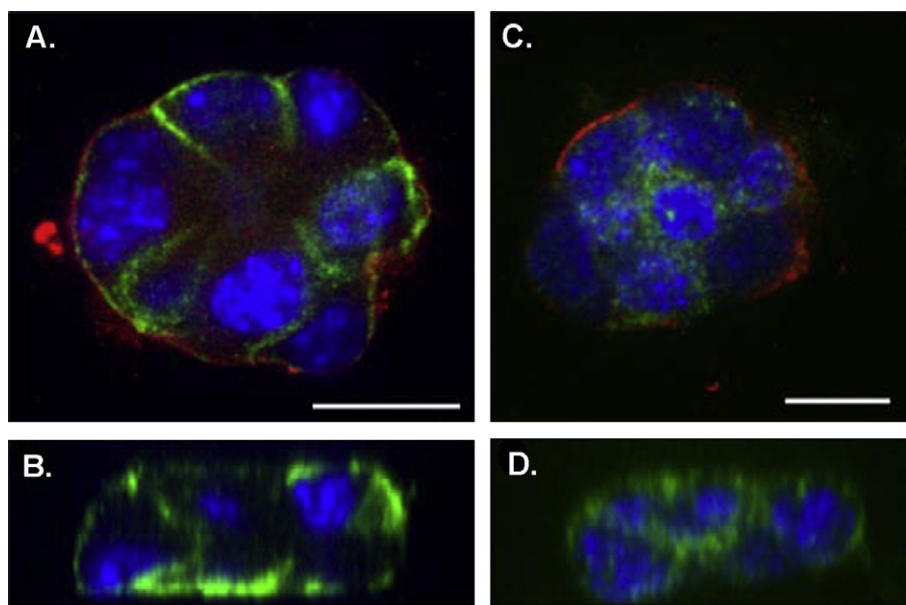

Fig. 3. Localisation of PMCA1 and PMCA2 in SCp2 cells grown in 3D culture. (A) A representative acinus showing staining for PMCA1 (green), α6 integrin (red), and DAPI (blue). (B) The corresponding z–x plane through the acinus described in A. (C) A representative acinus showing staining for PMCA2 (green), α6 integrin (red), and DAPI (blue). (D) The corresponding z–x plane through the acinus described in C. Scale bars represent 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

exhibited pronounced differences in their sub-cellular localization in SCp2 cells. PMCA1 expression was basolateral (Fig. 3A and B and supplementary Fig. 2), whereas PMCA2 localization was more predominant towards the apical membrane and exhibited a cytosolic punctate staining (Fig. 3C and D and supplementary Fig. 3).

Discussion

The modest increase in SPCA1 expression compared with PMCA2 expression during lactation in the rat [5,8], and the reduction of the Ca^{2+} content of milk in $PMCA2^{-/-}$ mice [9], has challenged the dogma of the greater importance of the Golgi compared to the apical membrane in the enrichment of milk with Ca^{2+}. The data presented here suggest that SPCA2, rather than SPCA1, is the major secretory pathway Ca^{2+} ATPase important in lactation and is consistent with a major role of the Golgi in Ca^{2+} enrichment in milk.

The role of SPCA2 in mammary gland secretory cells during lactation is likely to be 2-fold: firstly, the up-regulation of SPCA2 may function to supply the secretory pathway with Ca^{2+} ions for packaging into casein micelles for calcium secretion into milk [4]. Indeed, the higher enzymatic turnover rate of SPCA2 compared to SPCA1 when over-expressed in HEK-293 cells and its high apparent affinity for Ca^{2+} [20], suggests that SPCA2 has an enhanced capability to replenish Golgi Ca^{2+} as Ca^{2+} is lost via secretion [20]. Since milk has a high Ca^{2+} content, lactation is an event where loss of Ca^{2+} from the tissue would be pronounced, and our observation of increased levels of SPCA2 during lactation is suggestive that this SPCA isoform has a major role in meeting the demand for Golgi Ca^{2+} replenishment. The second role for SPCA2 in lactation may go beyond the supply of Ca^{2+} to the Golgi for secretion into milk, and relate to the control of Ca^{2+} and Mn^{2+} ions in the Golgi for the regulation of Golgi-localized Ca^{2+} and Mn^{2+} sensitive enzymes, which are critical for post-translational modification of milk proteins and lactose production [3].

One key enzyme for which the regulation of Ca^{2+} and Mn^{2+} may be important is 1,4-β-galactosyltransferase I. This enzyme is a *trans*-Golgi enzyme [21] and is responsible for the transfer of galactose, a key event in the formation of some glycoconjugates [21,22]. 1,4-β-galactosyltransferase I is up-regulated during lactation, where it forms a heterodimer with α-lactalbumin to enable lactose production for secretion into milk [21]. The increase in both 1,4-β-galactosyltransferase I and SPCA2 during mid-pregnancy [21], combined with the sensitivity of 1,4-β-galactosyltransferase I to Mn^{2+} [23] suggests that SPCA2 may also be important in regulating the activity of this enzyme, and subsequently the lactose and glycoprotein profile of milk. The up-regulation of SPCA2 during mid-pregnancy is likely to be hormonally regulated as SPCA2 transcription is increased by prolactin in human MCF-7 breast cancer cells [24] and prolactin levels increase during pregnancy [25].

In addition to highlighting a physiological role for SPCA2 in lactation, this study provides further evidence for the isoform specific roles of PMCA2 and PMCA1 in the mammary gland, through the demonstration of their distinct sub-cellular and cellular locations during lactation. As expected, the ubiquitously expressed PMCA1 was present in the majority of cells in the lactating mammary gland with a basolateral localization both in vivo during lactation and in 3D culture. Such a distribution of PMCA1 is consistent with a role in the regulation of calcium fluxes in mammary gland epithelial cells, rather than direct transport of calcium into milk. Conversely, PMCA2 was localized to the secretory alveolus in vivo, with an apical localization, which is consistent with its proposed role in direct apical transportation of Ca^{2+} ions into milk [9,10]. PMCA2 localization in the 3D culture model appeared more pronounced towards the apical membrane, however, a vesicular distri-

bution was also apparent, suggestive of a high turnover due to loss of PMCA2 during lactation as previous proposed [8], or indicative of a role for PMCA2 in the accumulation of Ca^{2+} into secretory vesicles [9] during particular periods of lactation. The reduction of total milk Ca^{2+} content by 60% in PMCA2 null mice, combined with the major contribution of casein micelles in milk Ca^{2+} enrichment, could also suggest a role for PMCA2 in Golgi Ca^{2+} accumulation [9]. However, the observation that PMCA2 heterozygous mice do not have reduced milk Ca^{2+} content, despite a 38% reduction in mammary PMCA2 levels [9], and our observation of pronounced up-regulation of SPCA2, suggests that more complex reasons may exist for the pronounced reduction of Ca^{2+} milk content in PMCA2 null mice, and that this result does not imply that 60% of Ca^{2+} transport into milk is mediated via PMCA2. SPCA1-null mice have only been characterized recently and are embryonic lethal [26]. SPCA2-null mice have not been characterized; however, we would predict from our studies that their phenotype may be associated with altered calcium secretion into milk, and an altered lactose content and glycoprotein profile of milk.

Despite our increasing knowledge of the transport of Ca^{2+} during lactation, important questions still remain. Our studies have, however, helped to clarify and highlight the importance of the secretory pathway ATPase, SPCA2, during pregnancy and lactation. They have also focused attention on the specific roles of the PMCA isoforms, PMCA1 and PMCA2 suggested by their different cellular and sub-cellular localization in the lactating mouse mammary gland.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.03.003.

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