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The Ca^{2+}/H^{+} antiporter TMEM165 expression, localization in the developing, lactating and involuting mammary gland parallels the secretory pathway Ca^{2+} ATPase (SPCA1)

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ABSTRACT

Plasma membrane Ca^{2+}-ATPase 2 (PMCA2) knockout mice showed that ~60% of calcium in milk is transported across the mammary cells apical membrane by PMCA2. The remaining milk calcium is thought to arrive via the secretory pathway through the actions of secretory pathway Ca^{2+}-ATPase's 1 and/or 2 (SPCA1 and 2). However, another secretory pathway calcium transporter was recently described. The question becomes whether this Golgi Ca^{2+}/H^{+} antiporter (TMEM165) is expressed sufficiently in the Golgi of lactating mammary tissue to be a relevant contributor to secretory pathway mammary calcium transport. TMEM165 shows marked expression on day one of lactation when compared to timepoints prepartum. At peak lactation TMEM165 expression was 25 times greater than that of early pregnancy. Forced cessation of lactation resulted in a rapid ~50% decline in TMEM165 expression at 24 h of involution and TMEM165 expression declined 95% at 96 h involution. It is clear that the timing, magnitude of TMEM165 expression and its Golgi location supports a role for this Golgi $Ca2^{+}/H^{+}$ antiporter as a contributor to mammary Golgi calcium transport needs, in addition to the better-characterized roles of SPCA1&2.

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1. Introduction

Calcium concentration in milk ranges between 30–80 mM [1–3] compared to 2–2.5 mM in blood. Many Ca^{2+} transporters, channels, and binding proteins are required to coordinate calcium transport into, through and out of the mammary epithelial cell into milk while maintaining submicromolar mammary epithelial cell Ca^{2+} to avoid Ca^{2+} mammary epithelial cell toxicity and cell death [2–12]. The current model of mammary epithelial cell Ca^{2+} transport is evolving rapidly. Recently experiment evidence showed that polarized mammary epithelial cell take up Ca^{2+} via Store-independent Ca^{2+} entry (SICE), at the basolateral membrane by an unconventional interaction between the second isoform of the Golgi secretory pathway Ca^{2+}-ATPase (SPCA2), and the Ca^{2}+ influx channel Orai1 [4]. Ca^{2+} entering the mammary epithelial cell then travels one of two possible cellular transport paths to arrive in milk. In the first transport path (accounting for ~40% of calcium in milk) Ca^{2+} entering mammary epithelial cell via Orai1/SPCA2 route is likely rapidly sequestered in Golgi/secretory stores via SPCA1 and/or SPCA2 where it is bound to casein and facilitates casein micelle formation. Ca^{2+}-casein is packaged in secretory vesicles and secreted into milk as Ca^{2+}-casein [2,7,9,13,14]. The second transport pathway (accounting for ~60% of calcium in milk) involves cell Ca^{2+} that is pumped directly across the mammary epithelial cell apical membrane into milk by PMCA2bw [11,15]. The proteins involved in sequestering mammary epithelial cell Ca^{2+} while it is shuttled from the basolateral membrane to PMCA2bw are unknown.

Calcium transport into, through the lactating mammary epithelial cell and into milk is enormous [1–3] with Ca^{2+} playing a critical role in regulation of many aspects of cell function and cell life [16]. Therefore, it is not unexpected that a large number of expressed genes will be required to control the massive transcellular mobilization of Ca^{2+} from the blood into milk by the coordinated action of Calcium Transporters, including pumps, channels, sensors and buffers, in a functional module termed CALTRANS [3]. The critical role of the Golgi in mammary epithelial cell Ca^{2+} transport along with the recent evidence that TMEM165 is a Golgi Ca^{2+}/H^{+} antiporter [17] suggested that TMEM165 could be another member of the mammary epithelial cell CALTRANS module. Examination of lactation gene expression experiments in the Gene Expression Omnibus at NCBI [18] showed that TMEM165 mRNA expression was positively correlated with lactation and mRNA was upregulated ~10

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times over prepartum levels [19,20] (see: http://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS2843:160549_at and http://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS2360:160549_at).

In the present study we show that TMEM165 is Golgi located and its expression is upregulated ~25 times in lactation and downregulated ~95% in involution which parallels the location and pattern of expression of Golgi calcium pump SPCA1 in mammary tissue [4,9,10].

2. Materials and methods

2.1. Animals

The National Animal Disease Center's Animal Care and Use Committee approved all animal procedures. Lactating 129/SV mice were housed individually, in hanging basket cages on sawdust bedding. All mice were equalized to 6 pups per mouse mother on day one of lactation. Mice were euthanized at 10, 7, 3 and 1 days prepartum and 1, 3, 6, 12 and 18 days postpartum for tissue collection. Involution was initiated by removal of pups on day 12 of lactation for some mice. Starting on day 12 of lactation (time zero of involution), mice were euthanized at time zero, 24, 48, 72 and 96 h of involution. Mice were euthanized with a 50:50 mix of $C0_2$:O_2 followed by decapitation. Mammary tissue was removed, flash frozen in liquid N_2, and stored at −70 °C until membranes were prepared.

2.2. Mammary tissue microsomes

Microsomes were prepared as previously described [11]. Briefly, tissue was homogenized in 10 volumes of Buffer A which contained; Tris–HCl (10 mM), $MgCl_2$ (2 mM), EDTA (1 mM), protease inhibitor cocktail (Sigma, St. Louis, MO) at pH 7.5. The homogenate was mixed with an equal volume of Buffer B (Buffer A plus 0.3 M KCl) and centrifuged at 4000*g* for 10 min. The supernatant was collected, adjusted to 0.7 M KCl by the addition of solid KCl, and centrifuged at 100,000*g* for 1 h. The supernatant was discarded and the pellets were resuspended in Buffer C (Buffer A plus 0.15 M KCl). Membrane preparations were stored at −70 °C until assayed. Microsomes from PMCA2 wild type and knockout mice were prepared exactly as described [11].

2.3. Gel electrophoresis and Western blotting

The methods were basically as described previously [4,11]. Microsomes from mammary tissue were incubated for 15 min at room temperature in a modified Laemmli buffer containing 150 mg/ml urea and 65 mM DTT. Samples were then electrophoresed for 50 min at 200 V in a 4–12% Novex NuPAGE® Bis-Tris Gel using MOPS SDS running buffer (Life Technologies, Grand Island, NY). Proteins were transferred to nitrocellulose membranes using the iBlot Dry Blotting System (Life Technologies, Grand Island, NY) at 23 V for 7 min. The blots were blocked with StartingBlock T20 (Thermo Fisher Scientific Inc.). TMEM165 (Proteintech, Chicago, IL), SPCA1 (RS-1 #227) or SPCA2 (orange #2) antibodies were diluted 1/2000 in StartingBlock T20 and the blots were incubated over night at 4 °C. After washing they were incubated 1 h at RT with 1/50000 HRP goat anti-rabbit #31460 (Thermo Fisher Scientific Inc.) and washed. Blots were developed using Pierce's Supersignal (Pierce Products, Rockford, IL) using the protocol provided by the manufacturer. Developed film was imaged and bands quantitated with a Gel Doc EZ imager (BioRad, Hercules, CA). Proteins were determined using the BioRad Protein Assay Kit using a BSA standard. Anti-SPCA2, and SPCA1 were described previously [4,9,10].

2.4. Confocal immunofluorescence microscopy

Tissue samples were prepared for and immunofluorescence microscopy was performed as described [4]. Primary antibodies used were anti-TMEM165-aminoterminal end (Abcam, Cambridge, MA) 1/100 anti-GM130 #610822 (BD Transduction Laboratories, San Jose, CA). SPCA1 (1/100) and SPCA2 (1/100) antibodies are described above. Secondary antibodies were (1:1000) Green anti-mouse Alexa Fluor A11017 488 $F(ab')_2$ fragment of goat anti-mouse IgG(H + L), and Red anti-rabbit at 1:1000 Alexa Fluor A11070 594 $F(ab')_2$ fragment of goat anti-rabbit IgG (H + L) (Molecular Probes/Life Technologies, Grand Island, NY). Slides were viewed and photographed on Zeiss LSM 5 PASCAL Axioplan 2 Imaging (Carl Zeiss, Germany).

2.5. Statistics

Statistics were done using the JMP statistical package (SAS Institute Inc. Cary, NC). Replicates were $n = 4–5$ mice and the data are presented as means ± SEM.

3. Results

The patterns of TMEM165 protein expression in mammary tissue of mice at 11 days of pregnancy to 18 days of lactation were examined by Western blot. Fig. 1A shows that TMEM165 protein expression was up significantly ($P < 0.05$) by 3 days prepartum and its expression peaked at day 12 of lactation at ~25 times ($P < 0.01$) the level of expression seen in early pregnancy. Forced mammary involution due to abrupt cessation of lactation on day 12 is associated with rapid accumulation of mammary calcium and a rise in blood calcium [10] which correlates in part with ~50% decline ($P < 0.01$) in TMEM165 expression 24 h of involution and TMEM165 expression declined 95% at 96 h involution (Fig. 1B).

In previous work we had shown that loss of the primary mammary epithelial cell calcium pump PMCA2bw was associated with significant compensatory upregulation of other calcium transporters such as SPCA1 and SERCA2 [11] TMEM165 expression was shown to be significantly ($P < 0.01$) up in PMCA2 knockouts compared to wild type expression (Fig. 1C) which is suggestive of a calcium transport role for TMEM165 in mammary epithelial cell lactation.

Fig 2 shows that TMEM165 co-localizes with the Golgi marker, GM130, (top panels) which is nearly identical to how SPCA1 co-localizes with the Golgi marker, GM130 (middle panels) in lactating mammary tissue. SPCA2 (bottom panels) in contrast has a diffuse vesicular distribution, with little co-localization with Golgi marker GM130. This is in marked contrast to the similarities between TMEM165 and SPCA1 mammary localization. Finally Fig. 3 shows mammary TMEM165 staining with GM130 at selected times during pregnancy, lactation and involution which are in agreement with Western blot data (Fig. 1).

4. Discussion

Lactating mammary cell calcium homeostasis is maintained by the high expression of SPCA1, SPCA2 and PMCA2, which move and control the enormous amounts of calcium required for milk production into storage compartments such the Golgi or out to milk [4,9–11,21]. The molecular identity of additional calcium transport pathways in lactating mammary epithelial cell remains unknown. Genome-wide screens [14] have been used in an attempt to reveal additional Calcium Transporters, including pumps, channels, sensors and buffers, in a functional module termed CALTRANS [3]. However, the success of microarray or transcriptomics approaches