Biochemical and Biophysical Research Communications 378 (2009) 99–102

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mammary gland involution is associated with rapid down regulation of major mammary Ca^{2+}-ATPases

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ARTICLE INFO

Article history:
Received 3 November 2008
Available online 10 November 2008

Keywords:
Ca^{2+}-ATPase
Lactation, mammary gland involution
Milk
PMCA
SERCA2
SPCA1
SPCA2
Mammary calcium

ABSTRACT

Sixty percent of calcium in milk is transported across the mammary cells apical membrane by the plasma membrane Ca^{2+}-ATPase 2 (PMCA2). The effect of abrupt cessation of milk production on the Ca^{2+}-ATPases and mammary calcium transport is unknown. We found that 24 h after stopping milk production, PMCA2 and secretory pathway Ca^{2+}-ATPases 1 and 2 (SPCA1 and 2) expression decreased 80–95%. PMCA4 and Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase 2 (SERCA2) expression increased with the loss of PMCA2, SPCA1, and SPCA2 but did not increase until 72–96 h of involution. The rapid loss of these Ca^{2+}-ATPases occurs at a time of high mammary tissue calcium. These results suggest that the abrupt loss of Ca^{2+}-ATPases, required by the mammary gland to regulate the large amount of calcium associated with milk production, could lead to accumulation of cell calcium, mitochondria Ca^{2+} overload, calcium mediated cell death and thus play a part in early signaling of mammary involution.

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Intracellular calcium ions (Ca^{2+}) regulate many aspects of cell function and cell life[1]. In the lactating mouse, movement of Ca^{2+} from 1.5 ml blood compartment (~1.2 mM Ca^{2+}) through the mammary secretory cells (1×10^6 mM Ca^{2+}) to secrete 10–12 ml of 80–100 mM calcium milk/day is a daunting task for the mammary cells calcium homeostatic mechanisms. Ionized calcium in mouse milk is similar to blood ionized calcium but the shear mass of calcium moved through the mammary cell/day equals the total calcium content of 200–400 ml of mouse blood [2–5].

The PMCA2, SPCA1, and SPCA2 are the most highly expressed calcium pumps in the lactating mammary gland of rat, mouse and cow [6–11]. About 60–70% of the calcium in milk arrives via PMCA2 mediated calcium transport from the cytosol, across the mammary cells apical plasma membrane and into milk [10,11]. The remaining calcium is thought to enter milk via a secretory process where SPCA1 and SPCA2 pump calcium into the Golgi where Ca^{2+} binds to casein, and Ca^{2+}-casein is secreted into the milk [6,8,10,12,13].

Mammary involution has been extensively studied and several comprehensive reviews have appeared recently [14–17]. Mammary involution is described as occurring as a reversible stage (~first 48 h), with extensive apoptosis followed by an irreversible stage with the dramatic tissue remodeling. The primary signal(s) that initiate mammary involution with abrupt cessation of lactation remain elusive [14] and a role for calcium as a potential trigger of involution has not been considered.

The calcium transport through lactating mammary cell is enormous. For any cell, loss of calcium homeostasis can lead to cell damage that can be either reversible or irreversible [2] much like reversible stage and irreversible stages of mammary involution. PMCA2, SPCA1, and SPCA2 are primarily responsible movement and regulation of mammary cell calcium and therefore cell calcium homeostasis. In the present study, we show that the expression of these major mammary Ca^{2+}-ATPases is reduced by 80–95% within 24 h of the start of mammary involution. This is associated with increase mammary calcium and precedes significant increases in other Ca^{2+}-ATPases, which might mitigate the losses of PMCA2, SPCA1 and SPCA2 on cell calcium homeostasis. These data suggest that loss of cell calcium homeostasis may be any early event in forced mammary involution.

Materials and methods

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. Involution was initiated by removal of pups on day 12 of lactation. Starting on day 12 of lactation (time zero of involution), mice were sacrificed at time zero, 24, 48, 72 and 96 h of involution. Mice were anesthetized with a 50:50 mix

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0006-291X/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.bbrc.2008.11.004

of CO_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.

Mammary tissue microsomes. Microsomes were prepared as previously described [10]. Briefly, tissue was homogenized in 10 volumes of Buffer A which contained; Tris–HCL (10 mM), $MgCl_2$ (2 mM), PMSF (0.1 mM), EDTA (1 mM), 4 μg/ml aprotinin, and 4 μg/ml leupeptin 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 4000g for 10 min. The supernatant was collected, adjusted to 0.7 M KCl by the addition of solid KCl, and centrifuged at 100,000g 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. Serum samples were taken for measurement of plasma calcium and $1,25(OH)_2D$ [18]. Mammary tissue was minced, washed in PBS, digested overnight in 40% Nitric acid in an autoclave and assayed for total calcium by Atomic absorption spectroscopy.

Gel electrophoresis and Western blotting. The methods were basically as described previously [10,13]. Briefly, microsomes 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 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 V in 0.7 M glycine, 0.025 M Tris at pH 7.4. Blots were developed using Pierce's Supersignal (Pierce Products, Rockford IL) using the protocol provided by the manufacturer. Proteins were determined using the BioRad Protein Assay Kit using a BSA standard. Anti-PMCA2, PMCA4, SPCA1, and SERCA 2 antibodies were described previously [8]. SPCA2 antibody was made in rabbits with peptide GTVCLLPSKEVIKGF-C using previously described methods [8]. Tubulin was used as a loading control.

Statistics. Statistics were done using the JMP statistical package (SAS Institute Inc. Cary, NC). Replicates were n = 5–6 mice and the data are presented as means ± SEM.

Results

Forced mammary involution, due to abrupt cessation of milk production on day 12 of lactation, is rapidly reflected in systemic measures of calcium homeostasis. Plasma calcium is up within 24 h of the start of mammary involution and is significantly elevated at 48 and 72 h into mammary involution (Table 1). The systemic calcium regulating hormone 1,25-dihydroxyvitamin D

Table 1
Effect of mammary involution on calcium homeostasis indicators and mammary calcium content.

	Hours of involution				
	0	24	48	72	96
Plasma calcium (mg/dl)	9.8 ± 0.3	10.5 ± 0.2	11.8 ± 0.3**	10.8 ± 0.3*	10.4 ± 0.2
Plasma $1,25(OH)_2D$ (pg/ml)	190 ± 12	36 ± 2**	40 ± 2**	26 ± 5**	82 ± 6**
Mammary gland calcium(μmol/g tissue)	5.4 ± 0.6	15.0 ± 0.2**	15.2 ± 0.3**	9.8 ± 0.9**	3.1 ± 0.4*

* Indicates significantly different ($P < 0.05$) from time zero within a row. Time zero = day 12 of lactation.
** Indicates significantly different ($P < 0.01$) from time zero within a row.

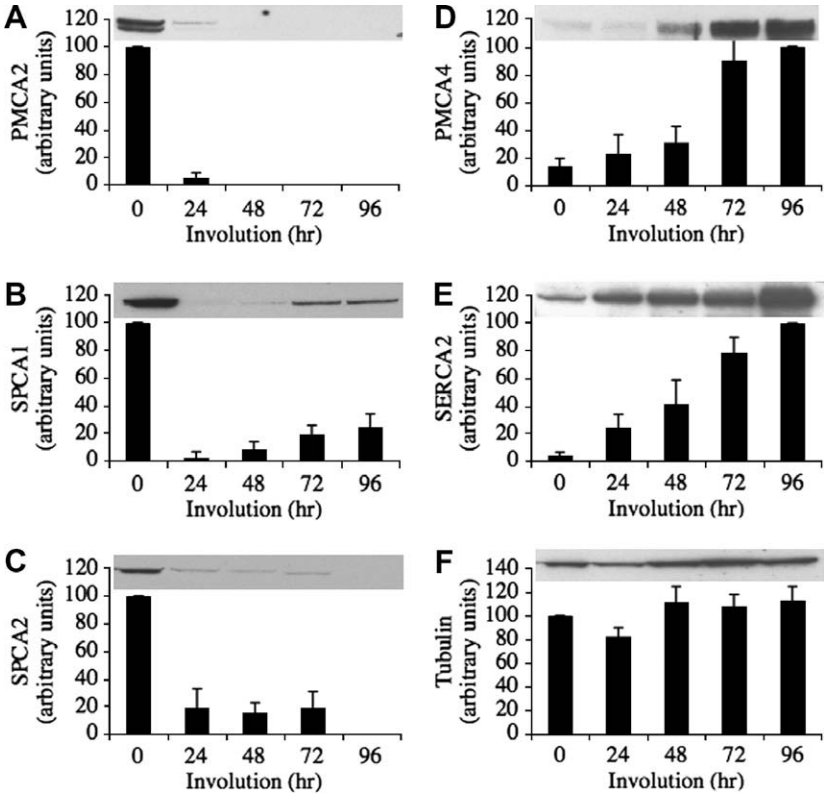

Fig. 1. Western blots and mammary microsome Ca^{2+}ATPase expression changes from time zero (day 12 of lactation) mammary involution through 96 h into mammary involution. PMCA2, SPCA1, SPCA2, PMCA4, and SERCA2 are presented in panel's (A)–(E), respectively. (D) Alpha tubulin expression which was use to normalize the data for sample loading differences. Data are means ± SE (n = 4–6 mice/time point).

($1,25(OH)_2D$) is an even more sensitive measure of the cessation of mammary calcium secretion as $1,25(OH)_2D$ levels in the plasma are significantly reduced 24–96 h into mammary involution (Table 1).

Fig. 1A–C shows that PMCA2, SPCA1, and SPCA2 expression declined 80–95% within 24 h of the start of mammary involution. PMCA2 expression was undetectable from 48–96 h into involution (Fig. 1A). In contrast, while SPCA1 (Fig. 1B) expression declined similarly to that seen for PMCA2, SPCA1 expression rebounded and at 96 h was 24% of its level at the start of involution. SPCA2 initial decline of 80% remained stable 72 h into involution and then became undetectable (Fig. 1C).

Coinciding with the significantly reduced expression of PMCA2, SPCA1, and SPCA2 was a significant increase in mammary calcium at 24–72 h into mammary involution (Table 1). This increased mammary calcium concentration started to decline 72 h into mammary involution, which corresponded with increased expression of PMCA4 and SERCA2 (Fig. 1D and E). Mammary calcium concentration dropped below time zero mammary calcium concentrations at 96 h into mammary involution or 24 h after PMCA4 SERCA2 expression peaked at 72–96 h of involution (Table 1 and Fig. 1D and E).

Discussion

Following the abrupt cessation of lactation, mammary involution enters its earliest stages described as a reversible stage (~first 48 h), with extensive apoptosis followed by an irreversible stage with the dramatic tissue remodeling. Despite the finding that 500 transcripts are upregulated within 12 h of involution [19], the consensus is that the primary signal(s) that initiate mammary involution with abrupt cessation of lactation are unknown local factors [20] and their identity remains elusive [14]. A role for calcium as a potential trigger of mammary involution has not been considered.

Lactating mammary cell calcium homeostasis is maintained by the high expression of SPCA1, SPCA2 and PMCA2, which move the large amounts of calcium required for milk production into storage compartments such the Golgi or out to milk [5,8,10,21]. The data present here show that all these calcium pumps are rapidly down-regulated early in involution Fig. 1A–C. In addition it is know that caspase activation is an early event in mammary involution [14] and caspase cleavage of PMCA's impairs intracellular Ca^{2+} handling that results in cell Ca^{2+} overload [22]. Loss of cell calcium homeostasis results in mitochondrial Ca^{2+} overload, enhanced generation of reactive oxygen species (ROS), triggering of the permeability transition pore and cytochrome *c* release, leading to apoptosis [23]. The observed systemic rise in blood calcium and decline in blood $1,25(OH)_2D$ at 24 h into involution further supports the loss of mammary calcium homeostasis as does the accumulation of mammary tissue calcium (Table 1).

Microarray data has suggested that mitochondria's role in mammary involution is primarily involved in the later stages of mammary involution [19]. In contrast the work by Stein et al. [16] suggests that mitochondria are responding to involution signals as early as 12 h into involution with the expression of ROS inducible stress genes. Others who have shown oxidative damage in mitochondrial DNA as early as 12 h into mammary involution support this work [24]. These later findings are consistent with loss of mammary cell calcium homeostasis and mitochondrial Ca^{2+} overload would likely result based on mammary loss of PMCA2, SPCA1, and SPCA2 expression. Furthermore, mammary mitochondria from lactating tissue have proven to be extremely sensitive to Ca^{2+} [25], which has prevented isolation of respiratory coupled mitochondria for lactation and involution studies [15].

Following the loss of PMCA2, SPCA1, and SPCA2 expression early in involution mammary tissue calcium remained high through 48 h of involution and only declined following marked increased expression of PMCA4 and SERCA2 at 72 h. Both of these calcium pumps are known to be expressed at low levels in lactation [8,9] and our data suggest they play more prominent role in cellular calcium regulation in the undeveloped or developing non-lactating mammary tissue [7].

The major mammary Ca^{2+}-ATPases are reduced by 80–95% within 24 h of the start of mammary involution. The data presented suggest that the abrupt loss of the major Ca^{2+}-ATPases, required by the mammary gland to regulate the large amount of calcium associated with milk production, could lead to accumulation of cell calcium, mitochondria Ca^{2+} overload and calcium mediated cell death. Therefore Ca^{2+} may be a one of the hypothesized local factor(s) [14,20] in early signaling of mammary involution though its role in early involution signaling requires additional study.

Acknowledgments

We thank Tera Nyholm, Adrienne Staple, Derrel Hoy and Duane Zimmerman for their excellent technical support and animal care in support of these studies.

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