

Review

## **CALCIUM TRANSPORT BY MAMMARY SECRETORY CELLS: MECHANISMS UNDERLYING TRANSEPITHELIAL MOVEMENT**

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**Abstract:** The secretion of calcium into milk by mammary epithelial cells is a fundamentally important process. Despite this, the mechanisms which underlie the movement of calcium across the lactating mammary gland are still poorly understood. There are, however, two models which describe the handling of calcium by mammary epithelial cells. On the one hand, a model which has existed for several decades, suggests that the vast majority of calcium enters milk via the Golgi secretory vesicle route. On the other hand, a new model has recently been proposed which implies that the active transport of calcium across the apical membrane of mammary secretory cells is central to milk calcium secretion. This short review examines the strengths and weaknesses of both models and suggests some experiments which could add to our understanding of mammary calcium transport.

**Key words:** Calcium, Mammary, Secretion

### **INTRODUCTION**

During lactation, mammary epithelial cells extract large quantities of ionized calcium from plasma and produce a calcium rich secretion. In doing so the mammary gland generates a large transepithelial calcium gradient in favour of milk [1]. Indeed, the concentration of calcium in milk can exceed 60 mM [2]. Not surprisingly, the transport and handling of calcium by the mammary

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Abbreviations used: PMCA - plasma membrane calcium-ATPase; SERCA – sarco/endoplasmic reticulum calcium-ATPase; SPCA – secretory pathway calcium-ATPase

epithelium has received considerable attention because of its importance in the context of neonatal nutrition. An understanding of calcium transport by mammary secretory cells is also important in relation to the integrity of mammary tight junctions and the synthesis of milk components such as caseins [3-5]. In addition, the potential link between calcium and breast cancer has increased the interest in mammary calcium transport [6, 7].

Unfortunately, the study of calcium transport by the mammary gland is complicated by the very nature of the secreted product. Milk calcium exists in a variety of forms including calcium bound to casein, colloidal calcium phosphate/citrate and free ionized calcium [8]. The majority of calcium is associated with casein micelles allowing milk to maintain calcium at levels which, if fully ionized, would put mammary secretory cells under considerable osmotic stress. The study of calcium transport by mammary tissue has also been hampered by the relatively complex anatomy of the gland. The morphology of the mammary gland makes the measurement of transepithelial calcium difficult because unlike some other epithelia it cannot be mounted in an Ussing chamber. The process of transepithelial calcium transport consists of at least three important steps [9]. The first is the transport of free ionized calcium across the blood-facing (i.e. basolateral) aspect of the mammary epithelium. The second step is the transfer of calcium across the cell which is a particularly challenging process for lactating mammary epithelial cells because they have to move large quantities of calcium in the blood-to-milk direction whilst keeping the concentration of free ionized calcium in the cytosolic compartment at a relatively low level. In common with other cell types, mammary epithelial cells have to maintain a low level of free calcium as failure to do so would compromise intracellular signalling and inevitably lead to cell death. The third step involves the movement of calcium across the apical membrane of the secretory epithelial cell and into milk.

A model based on experiments conducted several decades ago (hereafter referred to as Model 1) was formulated to describe the movement of calcium across the lactating mammary gland [8] (see Fig. 1). The major focus of Model 1 is the intracellular handling of calcium: it is envisaged that calcium is packaged within Golgi-derived secretory vesicles prior to its release via exocytosis into the milk space. Importantly, the model does not require the transport of free calcium across the apical membrane of the secretory cell. Recent data concerning the distribution of membrane CaATPases has, however, given rise to a new model (Fig. 2) which for the purposes of this review will be referred to as Model 2. This model proposes that the majority of calcium is directly pumped from the cytosol across the apical membrane into milk [10, 11]. This article reviews the experimental data which gave rise to both models and discusses their strengths and limitations. In addition, experimental approaches which could increase our understanding of mammary gland calcium transport are proposed.

### Model 1

The evidence in favour of model 1 is derived from biochemical, physiological and electron microscopical studies which were performed during the 1970s and 1980s [8, 9]. However, some aspects of the model are supported by recently published molecular and immunohistochemical data from the laboratories whose work has given rise to Model 2. The main features of Model 1 are: a) calcium which has been transported across the basolateral membrane of the secretory cell is actively pumped across the Golgi membrane b) the Golgi-derived secretory vesicles release the accumulated calcium into the lumen of the gland following exocytosis at the apical aspect of the cell c) no free calcium is transported across the apical membrane and d) the calcium concentration in milk is maintained as a consequence of the apical membrane being impermeable to calcium. It is assumed that calcium transport is transcellular with little or no paracellular movement [12].

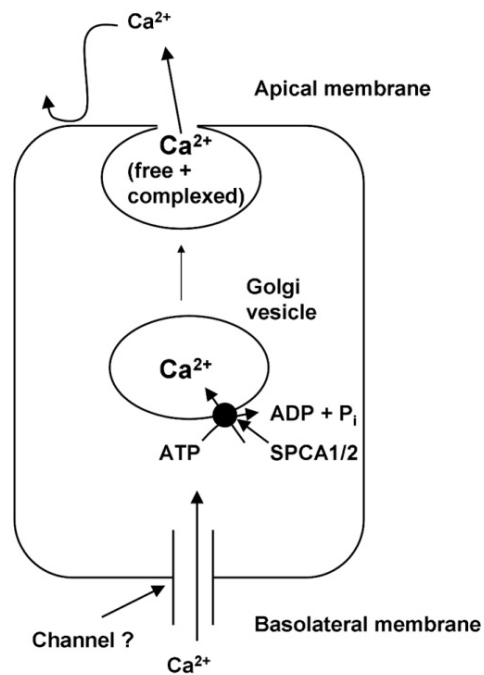


Fig.1. Model 1: the salient features of this model are 1) milk calcium is partitioned within Golgi vesicles prior to secretion 2)  $\text{Ca}^{2+}$  is not transported across the apical membrane from the cytosol into the lumen of the gland and 3) the apical membrane is relatively impermeable to milk calcium. There is an abundance of evidence supporting the presence of  $\text{Ca}^{2+}$ ATPase activity in Golgi-derived membranes [14-19]. Recent molecular studies suggest that SPCA1 and SPCA2 may fulfill this specialized role [20, 21]. Data from experiments which examined the appearance of radiolabelled calcium into milk, which had been injected close-arterially, underpins the suggestion that most of milk calcium is via the Golgi-secretory route [12]. Studies employing radiolabelled calcium suggest that the apical membrane does not display a high permeability to milk calcium [12].

The most salient feature of Model 1 is the prediction that milk calcium is partitioned within the cell and does not require calcium interacting with a transporter in the apical membrane. This part of the model is largely based on the study of Neville & Peaker [12] who measured the transfer of radiolabelled calcium across the lactating goat mammary gland. Two key findings emerged from this study. First, Neville & Peaker [12] found that the peak activity of  $^{47}\text{Ca}^{2+}$  administered close-arterially coincided with the secretion of casein into the lumen of the gland. The data of Neville & Peaker [12] appear to rule out a rapid, large trans-epithelial flux of free ionized calcium in the blood-to-milk direction. They concluded that milk calcium is partitioned within Golgi vesicles prior to secretion thus abrogating the need for calcium to directly cross the apical membrane [12]. Unfortunately, the long time courses employed in this set of experiments did not allow firm conclusions to be drawn about the precise nature of the pathways involved in the unidirectional transport of calcium across the lactating gland. Secondly, Neville & Peaker [12] found, in agreement with an earlier study [13], that a substantial portion of radiolabelled calcium introduced into the milk space of the lactating goat mammary gland remained after three hours suggesting that the apical membrane is relatively (but not completely) impermeable to ionized calcium. Thus, they found that 92% of a dose of  $^{47}\text{Ca}^{2+}$  administered to the lumen of the gland remained after 3 h. However, it is conceivable that such a high recovery of the lumenally administered radioactive  $\text{Ca}^{2+}$  could be due to a  $\text{Ca}^{2+}$ ATPase on the apical membrane acting to extrude calcium which had 'leaked' into the secretory cells. In addition, it is possible that radiolabelled calcium which crossed the apical membrane from the lumen of the gland may have been recycled into milk via the Golgi secretory route.

If the prediction of Model 1 that milk calcium is partitioned within the cell prior to secretion is correct, it follows that an active calcium transport mechanism would have to be associated with the Golgi membrane. In this connection there is ample evidence for ATP-dependent transport of  $\text{Ca}^{2+}$  by Golgi membranes isolated from lactating rat, mouse and bovine mammary tissue [14-18]. Golgi-derived membrane vesicles display ATP-dependent  $\text{Ca}^{2+}$  accumulation with an apparent  $K_m$  of 0.12-0.24  $\mu\text{M}$ . In accordance with this, Golgi membranes exhibit  $\text{Ca}^{2+}$ -dependent ATPase activity with kinetic properties similar to that of ATP-dependent  $\text{Ca}^{2+}$  uptake [17, 19]. However, it is notable that the  $\text{Ca}^{2+}$ ATPase activity is not confined to the Golgi membrane: it is apparent that microsomal membranes have the capacity to hydrolyse ATP in the presence of  $\text{Ca}^{2+}$  together with the ability to actively transport  $\text{Ca}^{2+}$  [16, 18]. However, the specific activity of the  $\text{Ca}^{2+}$ ATPase appears to be highest in the Golgi-enriched membrane fraction [16, 18]

Recent molecular studies appear to support the earlier functional observations that lactating mammary tissue expresses a  $\text{Ca}^{2+}$ ATPase in the Golgi-derived membrane fraction. Thus, mammary secretory cells express SPCA1 and SPCA2 both of which are specialized Golgi  $\text{Ca}^{2+}$ ATPases and could therefore be the molecular correlates of the  $\text{Ca}^{2+}$ ATPases. The presence of SPCA1 mRNA has

been reported in both rat and mouse mammary tissue, however, the level of expression increases only modestly during pregnancy and lactation suggesting that it may not play a significant role in the intracellular partitioning of milk calcium [20, 21]. On the other hand there is evidence to suggest that SPCA2 may be the most important isoform [21]. The expression of SPCA2 mRNA increased more than 35 fold in the mouse mammary gland during lactation [21]. In accordance with this, there is a preliminary report of SPCA2 mRNA expression in rat mammary tissue whose expression increases during lactation [22]. In addition, a recent study has suggested that prolactin stimulates  $\text{Ca}^{2+}$  entry into the Golgi apparatus via SPCA2 in MCF-7 cells [24]. The authors suggest that prolactin-induced SPCA2 activity may be physiologically relevant to the process of milk secretion. Although caution must be exercised when extrapolating findings made with cultured breast cancer cells to that of the lactating mammary gland the finding is consistent with a major role for SPCA2 in the transport of calcium by mammary cells.

Support for the transport of calcium by Golgi membranes can also be inferred from electron microscopy studies of lactating mammary tissue. Casein micelle formation requires the presence of significant amounts of calcium [25]. The association between calcium and casein appears to occur intracellularly as it is apparent that secretory vesicles contain electron dense material which is believed to be casein micelles (e.g. see [26]).

Although it is accepted that the process of calcium secretion into milk begins with calcium transport across the basolateral membrane of mammary epithelial cells, Model 1 does not provide any information about this important aspect. It has been predicted that channels may provide a high-capacity conduit for calcium across the basal pole of secretory cells [9, 11] but as yet there is no experimental evidence to support this idea. There is, however, indirect evidence for a swelling-induced calcium influx pathway in lactating mammary epithelial cells [27, 28]. Although this pathway has not been fully characterized it can be predicted, in common with other epithelial volume-activated calcium pathways, that it is a channel. Such a channel situated in the basolateral membrane of the epithelial cell could act to supply milk calcium. In addition, Model 1 does not take into account that a  $\text{Ca}^{2+}$  ATPase situated in the basolateral membranes may also be required to regulate intracellular  $\text{Ca}^{2+}$ . Recent data published by Faddy *et al.* [21] suggest that PMCA1 could fulfill this role. Thus, it has shown that PMCA1 has a basolateral location in mouse mammary epithelial cells [21].

### **Model 2**

Model 2 as proposed by VanHouten *et al.* [10, 11] is based on recent molecular and immunohistochemical studies. Although it incorporates some aspects of Model 1 it predicts that the majority of calcium is pumped across the apical aspect of the lactating mammary cell via a  $\text{Ca}^{2+}$  ATPase rather than relying solely on the Golgi vesicle secretory route. Another major feature of model 2 is that the association of calcium with casein micelles takes place predominantly in the

milk space of the gland (i.e. following secretion of casein). As mentioned above, it is generally believed that casein micelles are formed intracellularly prior to secretion. This view will have to be modified if it transpires that the majority of milk calcium is transported across the apical membrane by a  $\text{Ca}^{2+}$  ATPase.

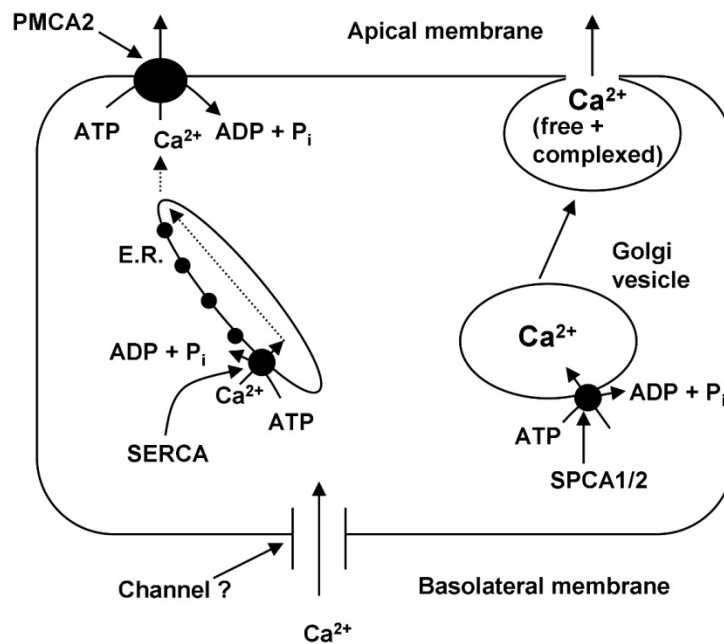


Fig 2. Model 2: the important features of this model are 1) calcium is actively pumped across the apical membrane 2) the ER may act as a conduit for calcium transfer across the cell and 3) milk calcium is also partitioned within Golgi secretory vesicles. It is envisaged that direct transport of calcium across the apical membrane by PMCA2 accounts for the majority of calcium in milk (60-70%) [10, 11, 32]. The remainder enters milk via the Golgi secretory route as depicted in Model 1. This model is largely based on molecular studies rather than on the measurement of radiolabelled calcium [10, 11, 20, 29, 32].

The first evidence that plasma membrane bound  $\text{Ca}^{2+}$  ATPases may be involved in calcium secretion came from the work of Reinhardt & Horst [20] who found that rat mammary tissue expressed mRNA for a number of plasma membrane  $\text{Ca}^{2+}$  ATPases and whose abundance changed with the transition from pregnancy to lactation. Thus mRNA for PMCA1, PMCA2 and PMCA4 were found in rat mammary tissue: the expression of each isoform increased between pregnancy and day 14 of lactation. However, PMCA2 was the most abundant transcript and exhibited the biggest change. In a follow up study, Reinhardt *et al.* [29] confirmed that rat mammary tissue expressed several  $\text{Ca}^{2+}$  ATPase proteins. Thus PMCA1, PMCA2 and PMCA4 proteins were identified: the amount of PMCA1 and PMCA2 protein increased between pregnancy and lactation with the latter being the most important quantitatively during lactation. On the other hand,

although PMCA4 mRNA increased during lactation in rat mammary tissue the amount of protein decreased. Reinhardt and colleagues have also provided evidence for the expression of PMCA2 and PMCA4 proteins in bovine mammary tissue [23].

The expression of PMCA2 and other  $\text{Ca}^{2+}$ ATPases in mammary tissue has recently been confirmed [10, 30]. However, there is an important difference between the study of VanHouten *et al.* [10] and those of Reinhardt and colleagues [20-29]. The latter group found that PMCA2 mRNA (and protein) did not increase in the rat until after parturition whereas VanHouten *et al.* [10] observed a large pre-partum increase in the expression of PMCA2 mRNA in the mouse mammary gland.

The notion that PMCA2 is involved in calcium transport across the apical membrane of mammary secretory cells arose from the finding that the  $\text{Ca}^{2+}$ ATPase is associated with the rat milk-fat-globule membrane [20]. It is generally held that the milk-fat-globule membrane is derived from the apical membrane during the exocytosis of lipid droplets from the secretory cell [31]. An apical location for PMCA2 in bovine mammary epithelial cells is also inferred from its presence on the milk-fat-globule membrane [23]. PMCA2 has also been located to the apical membrane by immunofluorescence and immunoelectron microscopy [10]. Interestingly, very little intracellular staining and immunoreactivity was found in lactating mammary secretory cells [10] which is surprising given that it can be predicted that there may be a large turnover of PMCA2 since significant quantities are lost by way of the milk-fat-globule membrane [31]. Nevertheless, there is sufficient evidence to suggest that PMCA2 is in a key position to play an important role in transepithelial  $\text{Ca}^{2+}$  transport. PMCA4 protein has also been shown to be associated with the bovine milk-fat-globule membrane which, according to the argument outlined above for PMCA2, suggests that it may also play a role in calcium transport across the apical membrane [23]. However, its importance may be related to calcium transport in and around the time of parturition given that the milk-fat-globule membrane levels of PMCA4 fall as lactation progresses [23].

A major apparent breakthrough regarding the importance of PMCA2 to mammary  $\text{Ca}^{2+}$  secretion came with the finding that a null mutation in the gene encoding PMCA2 markedly impaired the transport of calcium into mouse milk [32]. Indeed, milk from PMCA2-null mice had 60% less calcium compared to the milk of wild-type mice. Therefore, Reinhardt *et al.* [32] concluded that calcium transport across the apical membrane via PMCA2 was a fundamentally important step in the process of milk calcium secretion. VanHouten *et al.* [10] reached a similar conclusion on the basis that milk from mice, which exhibit mutations in the gene encoding PMCA2, have a lower milk calcium concentration. At first site, the evidence favouring PMCA2 as the major route for calcium transport across the apical membrane of mammary secretory cells is very convincing. However, the advocates for model 2 have made no attempt to reconcile their data with those of Neville & Peaker [12] regarding the time

course of calcium transfer across the lactating gland. Also, it is not beyond doubt that PMCA2 has a housekeeping role. Thus PMCA2 could act to extrude calcium which has 'leaked' back into mammary secretory cells from the gland lumen. A reduction in the capacity of mammary cells to extrude calcium, as in the case of a null-mutation, would of course have serious implications for mammary cell metabolism.

There is evidence suggesting that lactating mammary epithelial cells, in common with other cells, maintain cytosolic calcium at a low level [27, 28]. If active calcium transport via PMCA2 is the most important route for calcium movement across the apical membrane then it raises an important question: how do mammary secretory cells mediate a large net transepithelial calcium flux whilst maintaining a low intracellular calcium concentration? Calcium binding proteins could be involved in the process of shuttling calcium across the epithelial cell although at present there is no obvious candidate for this role [10, 11]. It has been suggested that calcium transport across mammary secretory cells may involve calcium sequestration by the endoplasmic reticulum near the basal aspect of the cell followed by release near the apical pole [10]. This would of course necessitate  $\text{Ca}^{2+}$ ATPases in the ER membrane. Indeed, rat mammary tissue appears to express both SERCA2 and SERCA3 mRNA the abundance of which is modestly affected by the physiological state [20]. Thus, the expression of SERCA2 and SERCA3 mRNA increased between pregnancy and lactation. VanHouten *et al.* [10] have confirmed the expression of SERCA2 and SERCA3 mRNA in mouse mammary tissue and have reported the presence of SERCA1 mRNA. However, VanHouten *et al.* [10] found that the level of SERCA mRNA, measured by microarray analysis, either decreased (SERCA1) or increased only slightly (SERCA2/3) as the animal progressed from pregnancy to lactation. Therefore, firm conclusions regarding the importance of the endoplasmic reticulum as a conduit for milk calcium cannot be drawn from the available data. As with Model 1, Model 2 does not provide any insight about the mechanism of calcium transport across the basolateral membrane of mammary secretory cells. Whatever the mechanism(s), it has to be one of high capacity to supply the epithelial cell with sufficient calcium to support lactation. As with Model 1, calcium channels are implicated [11].

### **Future trends**

Our understanding of mammary calcium transporters at the molecular level has increased markedly over the last decade and has given rise to an alternative model of mammary calcium transport. However, molecular studies alone are insufficient to support an acceptable model, therefore, it will be necessary to study calcium transport by the mammary epithelium.

One of the major claims of Model 2 is that most of milk calcium is transported across the apical membrane of the secretory cell by PMCA2. However, despite the importance of this point it remains to be shown that calcium is actively transported across the luminal membrane. In this connection there are methods



available which would allow apical membrane calcium transport to be studied with relative ease. Membranes isolated from milk, believed to originate from the apical membrane, could be used to study calcium transport. Thus, milk-fat-globule membranes and membranes isolated from the skim-milk fraction may be good experimental models. Conveniently, both membrane preparations form intact vesicles which can be used for transport studies [33, 34]. However, a study by the author has shown that membrane vesicles isolated from skim milk may be a better model than milk-fat-globule membranes to study apical membrane transport processes [33].

As mentioned above, neither model adequately accounts for the mechanism of calcium uptake across the basolateral membrane of mammary secretory cells. Isolated mammary tissue explants are probably the most convenient experimental model to study solute uptake across the blood-facing aspect of the gland. It is generally held that explants prepared from lactating mammary tissue give a measure of transport across the basolateral membrane of the secretory cells [9]. However, the presence of a large extracellular space rules out short time-course experiments. Therefore, explants as a means to characterize calcium transport across the basolateral aspect of secretory cells may be inadequate if calcium uptake, as predicted, is a rapid process. A better approach would be to use the perfused *in situ* mammary gland in conjunction with a rapid-paired tracer dilution technique. Such a method has been successfully used to characterize calcium transport across the dually perfused placenta [35]. Even though this method is semi-quantitative, it nevertheless allows transport to be characterized over very short time-courses under near physiological conditions. The only major drawback is that it is a technically difficult procedure. However, it has already been used successfully to study amino acid transport across the lactating rat mammary epithelium [36, 37]. The perfused mammary preparation could also be adapted to study transepithelial calcium transport. This would require the contents of the lumen to be analyzed following the administration of radiolabelled calcium to the perfusate.

Membrane vesicles prepared from the basolateral surface of mammary secretory cells would be another way to study the characteristics of calcium uptake. Basolateral membranes could be used to study the uptake of radiolabelled calcium under precise experimental conditions. In addition, basal membrane vesicles incorporated into lipid bilayers could be used to study the electrophysiological properties of putative calcium channels.

Cultured cells have proved invaluable to the study of mammary biology but they have serious limitations when applied to membrane transport processes underlying the secretion of milk. Cultured mammary epithelial cells are usually grown in media which has an ionic composition similar to that of plasma. Thus, the apical membrane of mammary cells will be exposed to a high rather than a low ionic strength environment: this will have implications regarding transepithelial electrical potentials which in turn could affect the polarity of membrane transporter distribution. In this regard, Quensell *et al.* [38] have

recently reported that the transepithelial resistance across cultured bovine mammary epithelial cells is markedly increased by exposing the luminal side of the epithelial cell layer to a low ionic strength buffer. The experimental model of Quensell *et al.* [37] could therefore be adopted to study calcium transport across the mammary epithelium. Such an experimental model would allow the transport of calcium to be measured simultaneously across the apical and basolateral aspects of the epithelium.

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