Polarized Expression of Ca$^{2+}$ Pumps in Pancreatic and Salivary Gland Cells

ROLE IN INITIATION AND PROPAGATION OF [Ca$^{2+}$] WAVES

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Min Goo Lee‡, Xin Xu‡, Weizhong Zeng‡, Julie Diaz‡, Tuan H. Kuo§, Frank Wuytack¶, Luc Ractymaekers∥, and Shmuel Mualem‡

From the ‡Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235, the §Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201, and the ¶Laboratory of Physiology, University of Leuven, Leuven, Belgium

The present study was aimed at localization of plasma membrane (PMCA) and intracellular (SERCA) Ca$^{2+}$ pumps and characterizing their role in initiation and propagation of Ca$^{2+}$ waves. Specific and polarized expression of Ca$^{2+}$ pumps was observed in all epithelial cells examined. Immunolocalization revealed expression of PMCA in both the basolateral and luminal membranes of all cell types. SERCA2a appeared to be expressed in the luminal pole, whereas SERCA2b was expressed in the basal pole and the nuclear envelope of pancreatic acini. Interestingly, SERCA2b was found in the luminal pole of submandibular salivary gland acinar and duct cells. These cells expressed SERCA3 in the basal pole. To examine the significance of the polarized expression of SERCA and perhaps PMCA pumps in secretory cells, we compared the effect of inhibition of SERCA pumps with thapsigargin and partial Ca$^{2+}$ release with ionomycin on Ca$^{2+}$ release evoked by agonists and Ca$^{2+}$ uptake induced by antagonists. Despite their polarized expression, Ca$^{2+}$ uptake by SERCA pumps and Ca$^{2+}$ efflux by PMCA resulted in uniform reduction in [Ca$^{2+}$]. Surprisingly, inhibition of the SERCA pumps, but not Ca$^{2+}$ release by ionomycin, eliminated the distinct initiation sites and propagated Ca$^{2+}$ waves, leading to a uniform increase in [Ca$^{2+}$]. In addition, inhibition of SERCA pumps reduced the rate of Ca$^{2+}$ release from internal stores. The implication of these findings to rates of Ca$^{2+}$ diffusion in the cytosol, compartmentalization of Ca$^{2+}$ signaling complexes, and mechanism of Ca$^{2+}$ wave propagation are discussed.

The agonist-evoked Ca$^{2+}$ signal is governed by the coordinated action of Ca$^{2+}$ channels and Ca$^{2+}$ pumps (1). In recent years it has become clear that [Ca$^{2+}$], changes occur in the form of elemental events emanating in specialized microdomains and propagating as Ca$^{2+}$ waves (see reviews in Ref. 2). Such behavior was also described in pancreatic acinar cells (3–6) in which the [Ca$^{2+}$], wave is initiated in the luminal pole (LP$^1$) and propagates to the basal pole. In the preceding manuscript (7) we showed similar phenomena in SMG acinar and duct cells. Characterization and localization of Ca$^{2+}$ release channels in the three cell types (7) suggests that the channels determine the initiation site and pattern of the [Ca$^{2+}$] waves. Thus, the cells express all three types of IP$_3$R and the SMG cells also express the rymodine receptor. Expression of the receptors appears to be confined to the area just underneath the luminal and lateral membranes (7).

The type of Ca$^{2+}$ pumps expressed in each cell type and their role in control of the [Ca$^{2+}$] wave is not known. Functional studies showed the presence of PMCA in pancreatic acini (8, 9), which is activated by agonist stimulation (8). In several studies isolated membranes and vesicles were used to localize the PMCA in the basolateral membrane of these cells (10). However, a recent study in which external [Ca$^{2+}$] was measured suggested that Ca$^{2+}$ pumping across the luminal membrane dominates Ca$^{2+}$ efflux in stimulated acinar cells (11). This predicts that the luminal membrane expresses high levels of PMCA.

As in many cell types, Tg and other inhibitors of SERCA pumps release Ca$^{2+}$ from internal stores of pancreatic acinar and SMG cells (8, 12). Again, the use of isolated endoplasmic reticulum vesicles shows the presence of Tg-sensitive Ca$^{2+}$ pumping activity in pancreatic acinar cells (10, 13). More recently an Ab raised against a C-terminal sequence of SERCA2b recognized two proteins of 111 and 97 kDa in pancreatic acini (14). Whether the cells express other SERCA isoforms and the localization of the SERCA pumps in pancreatic acini or SMG cells is not known.

In the present work we used WB analysis, immunocytochemistry, and rapid [Ca$^{2+}$], imaging to identify, localize, and study the function of the PMCA and SERCA pumps in controlling the Ca$^{2+}$ wave. Clear cell-specific and polarized expression of PMCA, SERCA2b, SERCA3, and SERCA2a were observed. Despite the high level of expression of PMCA in the luminal membrane, [Ca$^{2+}$], reduction in all parts of the cells occurred in the same rate. Completely unexpected was the finding that inhibition of the SERCA pumps with Tg abolished the initiation sites and led to a uniform and slow elevation in [Ca$^{2+}$], during agonist stimulation. These findings suggest a prominent role of the SERCA pumps in controlling the initiation site and propagation of the Ca$^{2+}$ wave.

MATERIALS AND METHODS

All methods pertaining to this manuscript are identical to those described in detail in the preceding manuscript including WB analysis.
immunocytochemistry using isolated cells or tissue slices, and Ca²⁺ imaging. The clone 5F10 recognizing all isoforms of PMCA and clone IID8 recognizing SERCA2 were purchased from Affinity Bioreagents (Golden, CO). SERCA2b-specific pAb were raised against a 12-amino acid C-terminal sequence of SERCA2b and characterized as described (15). pAb against SERCA2a were raised against a 9-amino acid C-terminal sequence of SERCA2a (16). Characterization of the SERCA3 pAb N89 was described before (17).

RESULTS

WB Analysis of PMCA and SERCA Pumps—WB analysis showed that all cell types express PMCA (Fig. 1). The Ab used detects all isoforms of PMCA (18), yet only one major band of about 140 kDa was found. This was the case when between 7.5 (underloading) and 75 µg (Fig. 1, overloading) of proteins were used.

Whether SM and pancreatic cells express SERCA2a, which is abundant in cardiac and slow skeletal muscle and to a lesser extent in smooth muscle and brain tissues (16, 17, 19), could not be determined with certainty by WB analysis. As shown in Fig. 1, the Ab against SERCA2a detected a faint band of about 95 kDa mainly in SMG acinar cells. A second band of about 55 kDa was also detected by these Ab. Similar results were found in four experiments. In two experiments the Ab detected only the 55-kDa band. This may suggest that if SERCA2a is expressed in SMG and pancreatic cells, it is expressed in low levels and is sensitive to proteolysis. Expression of SERCA2b in all cell types could be clearly demonstrated. Another Ab recognizing SERCA2b, mAb clone IID8, was used in the present studies. As the supplier indicated, this Ab did not recognize the rat SERCA2b by WB (not shown) but gave good signal in immunocytochemistry with pancreatic acini. Interestingly, only SMG duct and acinar cells expressed SERCA3. No such expression was found in pancreatic acini. To verify these results we also tested expression of SERCA2b and SERCA3 in the parotid gland. As was found in the pancreas, parotid acinar cells express SERCA2b but not SERCA3 (not shown). In addition, incubation of SMG duct and acinar cell extracts with pancreatic acini cell extract did not prevent detection of SERCA3 by WB.

Localization of PMCA and SERCA Pumps—Fig. 2 shows the pattern of expression of PMCA in pancreatic acini and SMG cells. Inspection of small pancreatic acinar clusters revealed the highest expression of PMCA in the luminal and lateral membranes. The doublet in the middle top image of Fig. 2 provides evidence for expression of PMCA in the luminal membrane. In other clusters labeling of the lateral membrane is also clear. In the top right image of Fig. 2 the basal membrane was brought into focus. Some punctate labeling of the basal membrane can be noticed. Probably the best evidence for expression of PMCA in the luminal membrane was obtained in SMG ducts (Fig. 2, bottom left image). The bottom right image of Fig. 2 shows the high levels of PMCA in the luminal membrane and the punctate pattern of expression in the lateral and basal borders. Expression of PMCA in the luminal membrane of SMG acinar cells can be seen in the bottom left image of Fig. 2.

Fig. 3 shows the polarized expression of SERCA2a and SERCA2b in pancreatic acini. Fig. 3 (A and B) show the exclusive staining of the LP of acinar cells with the SERCA2a Ab. This pattern is quite similar to the staining obtained with the different IP₃Rs Ab illustrated in the preceding manuscript (7). In most experiments low levels of staining could also be seen in the zymogen granule region (Fig. 3A). The highly localized and limited expression of SERCA2a may explain why it was difficult to detect expression of the pump by WB analysis. Pancreatic acinar cells express high levels of SERCA2b. The use of two different Ab showed that SERCA2b was expressed mostly in the basal pole. With both Ab, but especially with the mAb, clear staining of the nuclear envelope can be seen (Fig. 3C). Note that there was almost no labeling in areas distal to the nucleus, including the granular region. In four experiments with isolated acini and three experiments with pancreatic slices, there was no labeling of any cell type with the pAb specific for SERCA3.

Fig. 4 shows the localization of all SERCA pumps in SMG cells. Staining of SMG sections with a 1:500 dilution of SERCA2a revealed a highly specialized and patchy expression of SERCA2a in SMG acinar cells and complete absence of staining in the duct (Fig. 4A). Closer inspection of acinar structures (Fig. 4B) suggests that the labeling occurred in the small centroacinar cells (CAC). There was an impression that some labeling also occurred in the luminal membrane adjacent to the CAC. An attempt to better resolve this labeling by using higher Ab concentration failed because of increased background and/or the domination of the staining by CAC. Hence, at present we can only conclude that high levels of SERCA2a are expressed in what appears to be CAC.

The Ab against SERCA2b localized this pump to the LP of SMG duct and acinar cells. To clearly localize the SERCA2b,
high concentration of the pAb was required (1:100 dilution), which resulted in relatively low contrast images. Despite that, the pAb against SERCA2b stained exclusively the LP of duct cells with no staining of the BLP or the nuclear envelope. This was the case in cross-sections and longitudinal sections of the duct (Fig. 4D). In SMG acini SERCA2b was observed underneath the luminal and lateral membranes and over the nucleus. No staining in the basal region was observed. The results with the mAb were not conclusive, because of low signal/noise with this mAb in SMG cells (not shown). The pattern of expression of SERCA2b in SMG cells resembled the labeling obtained with all Ca$^{2+}$ release channels (7).

The use of isolated SMG acini (Fig. 4E) and duct fragments (Fig. 4F) suggested that SERCA3 is largely expressed in the basal pole of both cell types. In some duct cells labeling can also be seen to occur along the lateral membrane. In both cell types the labeling started in the basal membrane and continued to include at least some of the nuclear membrane. Hence, it seems that the pattern of SERCA3 expression in SMG cells was most like SERCA2b in pancreatic acini.

**Ca$^{2+}$ Pumps, Ca$^{2+}$ Transport, and Ca$^{2+}$ Waves**—To study the significance of the polarized expression of PMCA and the various Ca$^{2+}$ pumps, we followed [Ca$^{2+}$]$_i$ by rapid Ca$^{2+}$ imaging of single cells or small clusters of pancreatic and SMG acinar cells. The protocol designed to follow the activity of the PMCA and the SERCA pumps is illustrated in the bottom trace of Fig. 5 using a single pancreatic acinar cell. The cells were stimulated again to determine whether the cells to reduce [Ca$^{2+}$]$_i$ by rapid Ca$^{2+}$ imaging of single cells or small clusters of pancreatic and SMG acinar cells. The protocol designed to follow the activity of the

**FIG. 3. Localization of SERCA pumps in pancreatic acini.** Frozen sections of pancreatic slices (A and B) or isolated pancreatic acini (C and D) were stained with pAb against SERCA2a (A and B) or SERCA2b (D) or mAb against SERCA2b (C).

**FIG. 4. Localization of SERCA pumps in SMG cells.** SMG slices (A–D) or isolated cells (E and F) were stained with Ab specific for SERCA2a (A and B), SERCA2b (C and D), and SERCA3 (E and F).

**FIG. 5. SERCA pump activity regulates initiation site and rate of Ca$^{2+}$ release.** The entire experimental protocol for Figs. 5 and 6 is illustrated in the bottom left. The pancreatic acinar cell was stimulated with 100 μM carbachol (Car.) and at peak Ca$^{2+}$, inhibited with 1 μM atropine. The cell was then treated with 5 μM Tg for 120 s before a second stimulation with carbachol and inhibition with atropine. The bright field image in the top left indicates the luminal (LM) and basal (BLM) membranes, the areas in which [Ca$^{2+}$]$_i$ increase was analyzed (1, 2) and the direction of wave propagation. Selected images at 0.6-s intervals are shown for the periods of [Ca$^{2+}$]$_i$ increase during cell stimulation. The red and green traces show the changes in [Ca$^{2+}$]$_i$ in areas 1 (LP) and 2 (BLP), respectively, before and after Tg treatment.

**TABLE I**

| Treatment | Time to peak (s) | Ratio | P values |
|-----------|-----------------|-------|----------|
| Control  | 0.90 ± 0.07 (n=8) | 1.0 |          |
| Tg (5 μM) | 2.39 ± 0.46 (n=4) | 2.74 ± 0.86 | 0.0806 |
| BHQ (100 μM) | 3.58 ± 1.09 (n=4) | 4.48 ± 1.62 | 0.0078 |
| Overall  | 2.89 ± 0.58 (n=8) | 3.61 ± 0.91 | 0.0045 |

The rate of Ca$^{2+}$ release by 100 μM carbachol before (control) and after treatment with Tg or BHQ in the same cells were measured as in Fig. 5. The results are given as means ± S.E. p values for Tg or BHQ relative to control were determined by a paired Student’s t-test, and the overall p value was determined by analysis of variance.
The first and most notable finding was that inhibition of SERCA pumps resulted in elimination of the initiation site and propagation of Ca^{2+} wave. Rather, [Ca^{2+}]i increased uniformly in all parts of the cell. This was seen in all six experiments and eight cell studies and with single cells and cell clusters. To ensure that this effect was not a peculiarity of Tg, the experiments were also performed with 100 μM BHQ. Again, the same behavior was observed in three experiments and four pancreatic acinar cells. Another clear effect of inhibition of the SERCA pump was the marked reduction in the rate of Ca^{2+} release (i.e., the time needed for maximal [Ca^{2+}]i increase). This is illustrated in the traces in Fig. 5 and summarized in Table I. Treatment with BHQ seems to slow Ca^{2+} release somewhat better than Tg (4.48-fold compared with 2.74-fold, respectively). Inhibition of the SERCA pump reduced the rate of Ca^{2+} release by an average of 3.61-fold.

Fig. 6 shows the pattern of Ca^{2+} uptake by the SERCA (images and traces labeled before Tg) and the PMCA pumps (images and traces labeled after Tg). In both cases no cytosolic gradients of [Ca^{2+}]i were noted when the [Ca^{2+}]i was high, average, or low. This is illustrated in the images of Fig. 6, which shows images at selective time points, although fluorescence was recorded at high time resolution to obtain the traces in the bottom of Fig. 6. These findings were unexpected in the face of the polarized expression of all types of Ca^{2+} pumps (PMCA and SERCA) and the reported preferential extrusion of Ca^{2+} across the luminal membrane (11).

Because inhibition of SERCA pumps by Tg partially depleted internal stores and elevated resting [Ca^{2+}]i, prior to initiation of the wave, we tested whether similar effects induced by ionomycin and independent of pump inhibition would also result in elimination of the [Ca^{2+}]i wave. Fig. 7 shows that this was not the case. Modest (Fig. 7A) or marked (Fig. 7B) depletion of the pool and an increase in [Ca^{2+}]i beyond that induced by Tg (Fig. 5) did not prevent initiation and propagation of the [Ca^{2+}]i wave.

DISCUSSION

In the present work we attempted to determine the pattern of expression and role of PMCA and the SERCA pumps in initiation and propagation of the Ca^{2+} wave. Somewhat unexpectedly we found that PMCA was expressed both in the luminal and basolateral membrane of the three cell types studied (Fig. 2). Traditionally, PMCA is assumed to be expressed only in the basolateral membrane. Actually, isolation of membrane vesicles from many cell types, including pancreatic (10) and salivary glands (25) localized the PMCA to the basolateral membrane. However, most of these vesicle isolation techniques are not suitable for isolation of the luminal membrane, which is likely to be lost during membrane fractionation. On the other hand, a recent measurement of extracellular Ca^{2+} in small acini and single pancreatic acinar cells showed a more extensive Ca^{2+} efflux across the luminal membrane (11). The higher level of PMCA expression in the luminal membrane found in the present study can account well for these findings. The
identity of the PMCA isoforms expressed in the LM and BLM remains to be determined.

In view of the higher levels of PMCA in the luminal membrane (Fig. 2) and the high Ca$^{2+}$ efflux across this membrane in stimulated cells (see Ref. 11), we expected to see a faster rate of reduction in [Ca$^{2+}$], in the LP when SERCA pumps were inhibited. This was not the case. Rather, Ca$^{2+}$ efflux by PMCA resulted in uniform reduction in [Ca$^{2+}$], (Fig. 6, after Tg). The simplest explanation for this finding is that Ca$^{2+}$ diffusion in the cytosol is significantly faster than Ca$^{2+}$ efflux by PMCA. Indeed, efflux of all the released Ca$^{2+}$ by PMCA required 20–30 s, sufficient time for Ca$^{2+}$ to diffuse from the BLP to the LP (26).

Based on WB analysis and immunocytochemistry it is not possible to define all the isoforms of SERCA pump (or other proteins) expressed in the cells and their localization. However, this approach allowed us to demonstrate the first polarized expression of SERCA pumps in epithelial cells. Each of the cells studied appears to express at least two SERCA pump isoforms. All cells expressed the SERCA2b and SMG duct, and acinar cells express SERCA3. The picture with SERCA2a is less clear. The SERCA2a pAb were raised against a 9-amino acid C-terminal sequence of SERCA2a. A 5-amino acid sequence of this peptide are also found in SERCA2b at a distance of about 50 amino acids upstream of the C terminus (16). Hence, the possibility exists that the pAb against SERCA2a recognized some SERCA2b, although when tested no cross-reactivity of SERCA2a pAb with SERCA2b was found (16). In addition, the two Ab appear to recognize SERCA pumps in different poles of pancreatic acinar cells. Hence, when taken together the results point to the possibility that pancreatic acinar cells express SERCA2b and SERCA2a, whereas SMG acinar and duct cells express SERCA2b and SERCA3.

Interestingly, expression of the different SERCA pumps was highly polarized. Expression of SERCA2a is restricted to the LP and possibly the zymogen granule region of pancreatic acinar cells and SERCA3 to the basal pole of SMG duct and acinar cells. SERCA2b was expressed in the BLP of pancreatic acini and the LP and underneath the lateral membrane of SMG duct and acinar cells. Obviously, synthesis site and retention signals or sorting of SERCA2b must be cell-specific and different in the three cell types.

Measurement of [Ca$^{2+}$], in cells inhibited by atropine (when reduction in [Ca$^{2+}$], is mostly due to uptake by the SERCA pumps; see Refs. 8 and 20–24) did not reveal major differences in Ca$^{2+}$ uptake by the different SERCA pumps or at different regions of the cell. It is possible that Ca$^{2+}$ diffusion in combination with only small differences in the properties of Ca$^{2+}$ uptake by the different SERCA pumps (27) prevented detection of cellular Ca$^{2+}$ gradients at the spatial resolution of Ca$^{2+}$ recording of the present study. Hence, although at present the significance of the highly polarized expression of SERCA pumps is not known, it can be envisioned as contributing to the compartmentalization of the Ca$^{2+}$ pool and Ca$^{2+}$ signaling, to allow site-specific regulation of [Ca$^{2+}$].

Completely unexpected was the finding that SERCA pump activity controls the Ca$^{2+}$ wave initiation site and the rate of Ca$^{2+}$ release (Fig. 5). One possible explanation of these findings is that Tg treatment caused fusion of the compartmentalized Ca$^{2+}$ pools to convert quantal and localized Ca$^{2+}$ release (28, 29) to a continuous release from a single pool. This is considered unlikely because such an effect should not result in reduction in the rate of Ca$^{2+}$ release. In addition, preliminary experiments of localization of IP$_3$R in control and Tg-treated cells suggested that Tg had no effect on the polarized expression of IP$_3$Rs (not shown). Another possibility is that partial depletion of the stores and the increase in [Ca$^{2+}$], caused by Tg depleted the stores and therefore caused a desensitization of the Ca$^{2+}$ release channels. This explanation is excluded by the findings that treatment with low concentration of ionomycin failed to reproduce the effect of Tg.

The combined experiments with Tg and ionomycin strongly suggest that SERCA pumps actively participate in initiation and propagation of the [Ca$^{2+}$], waves. This can occur through the regulation of Ca$^{2+}$ release by endoplasmic reticulum Ca$^{2+}$ content (30) together with the heterogeneous affinity of the compartmentalized Ca$^{2+}$ pool to IP$_3$ (4, 29). Initiation sites are determined by high regions of IP$_3$R expression (7) with high affinity for IP$_3$ (4, 29). High local levels of [Ca$^{2+}$], during Ca$^{2+}$ release events allow Ca$^{2+}$ uptake by SERCA pumps into adjacent pools to increase their affinity for IP$_3$ (30) and trigger a second event of Ca$^{2+}$ release. By inhibiting the SERCA pumps Tg prevents the propagation of the Ca$^{2+}$ wave. At low [ionomycin] the [Ca$^{2+}$], increase adjusts to the site of [Ca$^{2+}$], release is sufficiently high to allow Ca$^{2+}$ uptake by SERCA pumps at this region and propagation of the Ca$^{2+}$ wave.

The most problematic aspect of the mechanism described above is that we could not find Ca$^{2+}$ release channels in any part of the cells, except underneath the luminal and lateral membranes and in the nuclear envelope (7). This raises the question of how [Ca$^{2+}$], increases uniformly in all parts of the cells without redistribution of Ca$^{2+}$ release channels. We do not have an immediate explanation for this problem. It is possible that the epithelial cells studied express very low levels of Ca$^{2+}$ release channels in all regions outside the LP below the detection sensitivity of the antibodies employed. It is also possible that Ca$^{2+}$ is released only in the cell periphery and that Ca$^{2+}$ diffusion is faster than Ca$^{2+}$ release in Tg-treated cells. In this case the upper limit of Ca$^{2+}$ diffusion rate should be close to but below the rate of the Ca$^{2+}$ wave in control cells, which is about 16.5 μm/s (Table I).

Localization of Ca$^{2+}$ transporters and measurements of [Ca$^{2+}$], waves in the present, and the preceding manuscript (7) demonstrates the specialized nature of the LP just underneath the luminal membrane in epithelial cells. It is likely that the high levels of PMCA in the luminal membrane, SERCA pumps, and Ca$^{2+}$ release channels in the LP form complexes of Ca$^{2+}$ transporters capable of controlling Ca$^{2+}$ in specific regions of the LP. These findings allow us to extend our proposal of compartmentalized Ca$^{2+}$ signaling complexes in acinar cells (6, 31). We now believe that Ca$^{2+}$ signaling complexes include the receptor, G protein, phospholipase C, and a PMCA, which are in close proximity and communication with specific portion of the endoplasmic reticulum containing specific SERCA pumps and a combination of Ca$^{2+}$ release channels. These complexes can operate autonomously to induce [Ca$^{2+}$], oscillations confined to part of the LP or communicate with neighboring complexes to trigger Ca$^{2+}$ waves along the cell periphery.

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