Baseline Cytosolic Ca$^{2+}$ Oscillations Derived from a Non-endoplasmic Reticulum Ca$^{2+}$ Store*

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Cytosolic Ca$^{2+}$ oscillations can be due to cycles of release and re-uptake of internally stored Ca$^{2+}$. To investigate the nature of these Ca$^{2+}$ stores, we expressed the Pmr1 Ca$^{2+}$ pump of Caenorhabditis elegans in COS-1 cells and pretreated the cells with thapsigargin to prevent Ca$^{2+}$ uptake by the sarco(endoplasmic reticulum Ca$^{2+}$-ATPase. Pmr1 co-localized with the Golgi-specific 58K protein and was targeted to a Ca$^{2+}$ store that was less leaky for Ca$^{2+}$ than the endoplasmic reticulum and whose inositol triphosphate receptors were less sensitive to inositol triphosphate and ATP than those in the endoplasmic reticulum. ATP-stimulated Pmr1-overexpressing cells responded after a latency to extracellular Ca$^{2+}$ with a regenerative Ca$^{2+}$ signal, which could be prevented by caffeine. They also produced very stable ilimaquinone-sensitive baseline Ca$^{2+}$ spikes, even in the presence of thapsigargin. Such responses never occurred in non-transfected cells or in cells that overexpressed the type-1 sarco(endoplasmic reticulum Ca$^{2+}$-ATPase. Abortive Ca$^{2+}$ spikes also occurred in histamine-stimulated untransfected HeLa cells pretreated with thapsigargin, and they too were inhibited by ilimaquinone. We conclude that the Pmr1-induced Ca$^{2+}$ store, which probably corresponds to the Golgi compartment, can play a crucial role in setting up baseline Ca$^{2+}$ spiking.

Many vital functions of a cell are controlled by periodic rises in the concentration of free Ca$^{2+}$ ions in the cytosol ([Ca$^{2+}$]), a phenomenon called cytosolic Ca$^{2+}$ oscillations. Many cells exhibit such oscillations, which are usually short-lived and intermittent, due to cycles of release and re-uptake of internally stored Ca$^{2+}$ (1). A few cells also exhibit sustained increases in the cytosolic Ca$^{2+}$ concentration due to an external store (2). These stores are usually large in size and can be activated by hormones and neurotransmitters. The release of Ca$^{2+}$ from these stores is often accompanied by a transient increase in the cytosolic Ca$^{2+}$ concentration due to the release of inositol 1,4,5-trisphosphate (IP$^3$) from the endoplasmic reticulum. IP$^3$ acts on the IP$^3$ receptor, which is an ion channel that is a member of the Ca$^{2+}$/H$^+$ exchanger family. The IP$^3$ receptor is located in the endoplasmic reticulum and can be activated by IP$^3$ or by other signals, such as diacylglycerol or cyclic AMP. The IP$^3$ receptor is a homo-oligomer of four subunits, each of which contains two Ca$^{2+}$ binding sites. The Ca$^{2+}$ binding sites are located in the cytoplasmic domain of the receptor and are involved in the regulation of the Ca$^{2+}$ permeability of the channel. The Ca$^{2+}$ binding sites are likely to be involved in the regulation of the Ca$^{2+}$ permeability of the channel. The Ca$^{2+}$ binding sites are likely to be involved in the regulation of the Ca$^{2+}$ permeability of the channel. The Ca$^{2+}$ binding sites are likely to be involved in the regulation of the Ca$^{2+}$ permeability of the channel. The Ca$^{2+}$ binding sites are likely to be involved in the regulation of the Ca$^{2+}$ permeability of the channel. The Ca$^{2+}$ binding sites are likely to be involved in the regulation of the Ca$^{2+}$ permeability of the channel. The Ca$^{2+}$ binding sites are likely to be involved in the regulation of the Ca$^{2+}$ permeability of the channel.
ity constants given by Fabiato and Fabiato (21). At the end of the experiment the \(^{45}\)Ca\(^{2+}\) remaining in the stores was released by incubation with 1 ml of a 2% sodium dodecyl sulfate solution for 30 min.

**Ca\(^{2+}\) Imaging**—\[^{45}\]Ca\(^{2+}\) was measured in single indo-1-loaded cells at 25 °C using an MRC-1024 (Bio-Rad) system as described (16). The cells were superfused with 135 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl\(_2\), 11.6 mM Hepes (pH 7.3), 11.5 mM glucose, and either 1.5 mM Ca\(^{2+}\) or 2 mM EGTA. Some cells were pretreated for 1 h with 2 \(\mu\)M thapsigargin.

**Microsomal Preparations**—Total microsomal fractions from non-transfected control COS-1 cells and from Pmr1- and SERCA-transfected COS-1 cells as well as from CHO cells were essentially prepared as previously described (22). CHO cells were chosen as positive control for IP\(_3\) receptor expression, because we previously reported the presence of the three IP\(_3\) receptor isoforms in those cells (23). Protein determination was performed

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**Fig. 1. Immunolocalization of Pmr1 in COS-1 cells.** A, co-localization of the Golgi-specific 58K protein and Pmr1 in COS-1 cells transfected with C. elegans Pmr1 expression vector. Primary incubation in Aa was with mouse antibody against the 58K protein (1/100), and secondary antibody was goat anti-mouse Alexa fluor 488 (Molecular Probes, dilution 1/500). Primary incubation in Ab was with rabbit anti-Pmr1 antiserum (1/10,000), and secondary antibody was goat anti-rabbit Alexa fluor 594 (Molecular Probes, dilution 1/500). Panel Ac shows the superimposed images. B, co-localization of the 58K protein and SERCA2a in COS-1 cells transfected with SERCA2a expression vector. Labeling of the 58K protein in Ba was as in Aa. Primary incubation in Bb was with rabbit anti-SERCA2a antiserum (1/500) and secondary antibody was goat anti-rabbit Alexa fluor 594 (dilution 1/500). Panel Bc shows the superimposed images.

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**Fig. 2. Ca\(^{2+}\) uptake and release in permeabilized COS-1 cells.** A, steady-state \(^{45}\)Ca\(^{2+}\) uptake (mean ± S.E., n = 4) by the non-mitochondrial Ca\(^{2+}\) stores of non-transfected control cells (open bars) and of Pmr1- (closed bars) and SERCA1-overexpressing cells (hatched bars) in the absence and presence of 2 \(\mu\)M thapsigargin (tg). B, passive Ca\(^{2+}\) leak from the Pmr1-induced Ca\(^{2+}\) store (●, full line) and from the endoplasmic reticulum (○, dotted line). Ca\(^{2+}\) uptake in the Pmr1-induced Ca\(^{2+}\) store was taken as the difference in Ca\(^{2+}\) uptake between non-transfected control COS-1 cells and Pmr1-overexpressing COS-1 cells in a medium containing 2 \(\mu\)M thapsigargin. Ca\(^{2+}\) uptake in the endoplasmic reticulum was taken as the Ca\(^{2+}\) uptake by control COS-1 cells in the absence of thapsigargin minus that in the presence of 2 \(\mu\)M thapsigargin. The Ca\(^{2+}\) content at time 0 of efflux was set at 100%. C, [IP\(_3\)] dependence of the IP\(_3\)-induced Ca\(^{2+}\) release from the Pmr1-induced Ca\(^{2+}\) store (●, full line) and from the endoplasmic reticulum (○, dotted line). Ca\(^{2+}\) release (means ± S.E. for n = 5) is expressed as a percentage of that induced by 10 \(\mu\)M of the Ca\(^{2+}\) ionophore A23187. The arrows point to the EC\(_{50}\) values for IP\(_3\)-induced Ca\(^{2+}\) release. D, [ATP] dependence of the IP\(_3\)-induced Ca\(^{2+}\) release. The Pmr1-induced Ca\(^{2+}\) store (●, full line) and the endoplasmic reticulum (○, dotted line) were stimulated with, respectively, 3 and 1 \(\mu\)M IP\(_3\) and the indicated [ATP]. Ca\(^{2+}\) release (means ± S.E. for n = 5) is plotted as a percentage of that observed in the absence of ATP, which was taken as 100%.
according to the Lowry procedure (24). All samples were quick-frozen in liquid nitrogen and stored until use at −80°C.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting Analysis—Microsomal preparations were boiled for 2 min in sample buffer, analyzed on 3–12% Laemmli-type linear gradient gels, transferred to Immobilon-P, and probed with isoform-specific antibodies against IP$_3$ receptor type 1 (Rbt04), type 2 (Rbt02), or type 3 (MMAType3, Transduction Laboratories, KY) (22, 25). The expression levels of Pmr1 and SERCA2b were measured in the same microsomal fractions using the previously described antibodies against Pmr1 (12) and SERCA2b (26).

Analysis of the immunoreactive bands after incubation with secondary antibodies coupled to alkaline phosphatase, detection using Vistra ECF (Amersham Pharmacia Biotech, UK) and fluorescence imaging were performed exactly as described before (27).

RESULTS

Overexpression of Pmr1 in COS-1 Cells—The 58K protein is a Golgi-specific protein (28). Fig. 1A shows a double labeling of the 58K protein (Fig. 1Aa) and Pmr1 (Fig. 1Ab) in COS-1 cells transfected with the cDNA of Pmr1 of C. elegans. The superimposed image (Fig. 1Ac) reveals that Pmr1 was mostly expressed in the same compartment as the 58K protein.
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Labeling of SERCA2a in COS-1 cells transfected with the cDNA of pig SERCA2a was used to immunolocalize the endoplasmic reticulum. Double labeling of the 58K protein (Fig. 1Ba) and SERCA2a (Fig. 1Bb) reveals that the subcellular distribution of SERCA2a differed from that of the Golgi marker (Fig. 1Bc).

Although these data clearly show that Pmr1 is targeted to the Golgi apparatus and has a different subcellular distribution than the endoplasmic reticulum marker, the technique of immunocytochemistry does not allow to prove that Pmr1 was exclusively present in the Golgi complex. Therefore, the functional properties of the Pmr1-induced Ca$^{2+}$ store were compared with those of the endoplasmic reticulum in control and SERCA-overexpressing cells.

Effect of Overexpressing Pmr1 on Intracellular Ca$^{2+}$ Uptake by COS-1 Cells—The ATP-driven uptake of Ca$^{2+}$ in non-mitochondrial Ca$^{2+}$ stores was measured in COS-1 cells with permeabilized plasma membrane. In non-transfected control COS-1 cells, Ca$^{2+}$ accumulation was fully inhibited by 2 $\mu$M thapsigargin (Fig. 2A, open bars), indicating that all this Ca$^{2+}$ uptake was SERCA-mediated and occurred in the endoplasmic reticulum. This conclusion is confirmed by the observation that the [Ca$^{2+}$], rise induced by addition of 100 $\mu$M of extracellular ATP as an agonist in intact non-permeabilized cells incubated in Ca$^{2+}$-free medium (Fig. 3A) also disappeared in the presence of thapsigargin (Fig. 3B).

When the Golgi-residing Pmr1 Ca$^{2+}$ pump of C. elegans was overexpressed in COS-1 cells, an additional thapsigargin-insensitive component of Ca$^{2+}$ uptake appeared in permeabilized cells (Fig. 2A, solid bars). An additional Ca$^{2+}$ uptake was also induced by transfection with SERCA1, which was, however, completely inhibited by 2 $\mu$M thapsigargin (Fig. 2A, hatched bars). The Pmr1-induced thapsigargin-insensitive Ca$^{2+}$ store was also available for agonist-induced Ca$^{2+}$ signaling, because it could be released by 100 $\mu$M extracellular ATP in intact non-permeabilized cells pretreated with 2 $\mu$M thapsigargin (Fig. 3C).

Properties of the Pmr1-expressing Ca$^{2+}$ Store in Permeabilized COS-1 Cells—Fig. 2B shows the passive leak of Ca$^{2+}$ from non-transfected control cells loaded with Ca$^{2+}$ in the absence of thapsigargin and from Pmr1-overexpressing cells loaded with Ca$^{2+}$ in the presence of 2 $\mu$M thapsigargin in an efflux medium containing 2 mM EGTA and no added Ca$^{2+}$ or ATP. Unidirectional Ca$^{2+}$ efflux occurred under these conditions, because (i) the calculated free [Ca$^{2+}$] (<10 nM) was below the threshold to stimulate the SERCA or Pmr1 Ca$^{2+}$ pumps and (ii) no ATP to fuel the pumps was included in the efflux medium. It is clear that the rate of Ca$^{2+}$ loss from the Pmr1-induced compartment (closed circles, full line) was much lower than that from the endoplasmic reticulum (open circles, dotted line).

To compare the [IP$_3$] dependence of the IP$_3$ receptors in the Pmr1-induced Ca$^{2+}$ store and in the endoplasmic reticulum, both types of stores were loaded with $[^{45}]$Ca$^{2+}$ and then challenged with IP$_3$ in efflux medium. The open circles and dotted line in Fig. 2C illustrate the Ca$^{2+}$ release from the endoplasmic reticulum as a function of the [IP$_3$]. The closed circles and full line are the values for the Pmr1-induced Ca$^{2+}$ store. The EC$_{50}$ was 1.1 $\mu$M IP$_3$ for the endoplasmic reticulum (dotted arrow) and 2.3 $\mu$M IP$_3$ for the Pmr1-induced Ca$^{2+}$ store (solid arrow).

A maximal [IP$_3$] released 88% of the ionophore-releasable Ca$^{2+}$ from the endoplasmic reticulum but only 33% from the Pmr1-induced Ca$^{2+}$ store. There are two possible explanations:

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**Fig. 5. Effect of overexpressing Pmr1 and SERCA1 on the Ca$^{2+}$ signals induced by 1 $\mu$M ATP in COS-1 cells.** A. Ca$^{2+}$ signals in 576 Pmr1-overexpressing cells pretreated with 2 $\mu$M thapsigargin. The numbers refer to the percentage cells producing baseline (a) or sinusoidal Ca$^{2+}$ oscillations (b), a single Ca$^{2+}$ peak (c), or a sustained [Ca$^{2+}$], rise (d). The gray bar in panel d represents the addition of 20 $\mu$M ilimaquinone. B, Ca$^{2+}$ responses in 1542 control cells that were not pretreated with thapsigargin. The percentage of cells producing baseline-like (a) or sinusoidal Ca$^{2+}$ oscillations (b), a single Ca$^{2+}$ peak (c), or a sustained [Ca$^{2+}$], rise (d) are given. 1% of the control cells did not respond. C, Ca$^{2+}$ responses in Coverglass chambers transfected with SERCA1 and not pretreated with thapsigargin. The percentage of cells producing baseline-like (a) or sinusoidal Ca$^{2+}$ oscillations (b), a single Ca$^{2+}$ peak (c), or a sustained [Ca$^{2+}$], rise (d) are given. (n = 315). 21% of the cells did not respond.
for this incomplete Ca\(^{2+}\) release. First, the IP\(_3\) receptors may be only expressed in a subcompartment of this store. Alternatively, feedback by the decreasing luminal [Ca\(^{2+}\)] could prevent complete Ca\(^{2+}\) release from a more homogeneous compartment, as has also been reported for the IP\(_3\)-induced Ca\(^{2+}\) release from the endoplasmic reticulum (20, 30).

Submillimolar ATP concentrations ensure a more efficient coupling between the IP\(_3\) binding at the N terminus of the IP\(_3\) receptor and the opening of the channel at its C terminus (31–34). Fig. 2D illustrates the effect of increasing concentrations of ATP on the IP\(_3\)-induced Ca\(^{2+}\) release from the Pmr1-induced Ca\(^{2+}\) store (closed circles, full line) and from the endoplasmic reticulum (open circles, dotted line). Ca\(^{2+}\) release from the former Ca\(^{2+}\) store differed from that in the endoplasmic reticulum by its insensitivity to ATP.

In conclusion, the Ca\(^{2+}\) store induced by overexpression of Pmr1 in COS-1 cells differed in its functional properties from the endoplasmic reticulum. These data are compatible with the different subcellular localization, as revealed by the immunochemistry in Fig. 1.

**Effect of Overexpressing Pmr1 on Capacitative Ca\(^{2+}\) Entry in COS-1 Cells**—Control and SERCA1- and Pmr1-overexpressing cells incubated in Ca\(^{2+}\)-free solution in the presence of 100 \(\mu\)M ATP dramatically differed in their response to re-addition of ATP.
1.5 mM extracellular Ca\(^{2+}\). The addition of extracellular Ca\(^{2+}\) to control COS-1 cells incubated in the presence of 100 \(\mu\)M ATP produced an immediate, but relatively slow rise in [Ca\(^{2+}\)], both in the absence (Fig. 3A) and presence of 2 \(\mu\)M thapsigargin (Fig. 3B). It was somewhat surprising that the addition of Ca\(^{2+}\) to control COS-1 cells stimulated with ATP gave a similar rise in [Ca\(^{2+}\)], in the presence and absence of thapsigargin, as the presence of the inhibitor was expected to result in a faster and larger Ca\(^{2+}\) signal because Ca\(^{2+}\) buffering by the endoplasmic reticulum would be inhibited. However, 300 \(\mu\)M IP\(_{3}\) released almost all of the releasable Ca\(^{2+}\) in permeabilized COS-1 cells (Fig. 2C), indicating that a maximal [ATP] resulted in the emptying of almost the entire endoplasmic reticulum Ca\(^{2+}\) store. SERCA1-overexpressing cells also responded to Ca\(^{2+}\) in the presence of thapsigargin with an immediate slow [Ca\(^{2+}\)] rise (Fig. 3D).

In contrast, Pmr1-overexpressing COS-1 cells stimulated with 100 \(\mu\)M ATP in the presence of 2 \(\mu\)M thapsigargin did not show an immediate response to re-addition of Ca\(^{2+}\), but reacted after a latency of variable duration with a sudden and fast [Ca\(^{2+}\)] rise, characteristic of a regenerative Ca\(^{2+}\) release (Fig. 3C).

The [Ca\(^{2+}\)] rise upon re-addition of Ca\(^{2+}\) to non-transfected control COS-1 cells in a medium containing 100 \(\mu\)M ATP and 2 \(\mu\)M thapsigargin was very similar in the absence (Fig. 4A) and presence of 10 mM caffeine (Fig. 4B), an inhibitor of the IP\(_{3}\) receptor (35–38). Inhibition of the IP\(_{3}\) receptor therefore had no effect on capacitative Ca\(^{2+}\) entry in COS-1 cells. In contrast, 10 mM caffeine prevented the regenerative [Ca\(^{2+}\)] rise in Pmr1-overexpressing cells, whereas washing out of the caffeine resulted in an immediate all-or-none [Ca\(^{2+}\)] rise (Fig. 4, C and D). This finding indicates that the IP\(_{3}\) receptor might be involved in the sudden and fast [Ca\(^{2+}\)] rise after the latency (see “Discussion”).

**Baseline Ca\(^{2+}\) Oscillations in Pmr1-overexpressing COS-1 Cells**—Immunofluorescence showed that about 30% of the transfected COS-1 cells expressed the worm Pmr1 (data not shown). These transfected cells were easily detectable during Ca\(^{2+}\)-imaging experiments, because only the transfected cells responded to ATP in the presence of thapsigargin (Fig. 3C), whereas non-transfected cells never responded under these conditions (Fig. 3B).

The majority (i.e. 57%) of the Pmr1-overexpressing COS-1 cells, when challenged with 1 \(\mu\)M ATP in the presence of 2 \(\mu\)M thapsigargin, set up remarkably stable baseline Ca\(^{2+}\) oscillations (Fig. 5A). The shape of the Ca\(^{2+}\) spikes differed among the cells (Figs. 5Aa and 6A). They could be abortive (Fig. 6B) or appear after a long latency of sometimes 10 min (data not shown). The frequency of the oscillation increased when the [ATP] was increased from 1 to 10 \(\mu\)M (Fig. 6C).

[Ca\(^{2+}\)], transients induced by 1 \(\mu\)M ATP in Pmr1-overexpressing cells persisted for some time in Ca\(^{2+}\)-free medium (Fig. 6D), indicating that the Ca\(^{2+}\) store was released from an intracellular source and that this Ca\(^{2+}\) store contained enough Ca\(^{2+}\), or was sufficiently efficient in Ca\(^{2+}\) re-accumulation, to set up a few [Ca\(^{2+}\)] rises. Sustained Ca\(^{2+}\) oscillations, however, depended on the presence of extracellular Ca\(^{2+}\) (Fig. 6D).

The Golgi-disturbing agent ilimaquinone (20 \(\mu\)M) profoundly influenced the Ca\(^{2+}\) spikes. Ilimaquinone could inhibit (Fig. 7A) or stimulate the oscillation induced by 1 \(\mu\)M ATP (Fig. 7B), or it could reversibly modify the shape of the spikes (Fig. 7C). Ilimaquinone could also convert a sustained [Ca\(^{2+}\)], rise in response to 1 \(\mu\)M ATP into a baseline Ca\(^{2+}\)-spiking pattern (Fig. 8).
Brefeldin A (5 μg/ml) was unable to mimic the effects of ilimaquinone (Fig. 7D). Baseline Ca\(^{2+}\) spiking persisted in 46% of the Pmr1-overexpressing COS-1 cells when the [ATP] was increased to 100 μM ATP (Fig. 8A).

Stable Baseline Ca\(^{2+}\) Oscillations Do Not Occur in Control COS-1 Cells—Endoplasmic reticulum-derived Ca\(^{2+}\) was not sufficient to support regular baseline Ca\(^{2+}\) spiking in COS-1 cells, because only 8% and 1% of non-thapsigargin-pretreated control cells responded with baseline-like spikes to, respectively, 1 μM (Fig. 5B) and 100 μM ATP (Fig. 8B); moreover, these oscillations always quickly decreased both in frequency and amplitude.

Ca\(^{2+}\) oscillations can often more easily be elicited in cells stimulated with low agonist concentrations (5). In control COS-1 cells, 0.2 μM ATP was a near-threshold concentration, because 37% of the cells did not respond to this low agonist concentration (Fig. 9). But also here, only 6% of the cells set up irregular low amplitude Ca\(^{2+}\) spikes when incubated in 0.2 μM ATP. The percentage of Pmr1-overexpressing cells that did not react to 0.2 μM ATP was similar (50%, data not shown), indicating that the sensitivity to ATP was not affected by overexpressing Pmr1.

Stable Baseline Ca\(^{2+}\) Oscillations Are Not Due to Increased Cellular Ca\(^{2+}\) Uptake per se—The non-mitochondrial Ca\(^{2+}\) stores in Pmr1-overexpressing cells stored twice as much Ca\(^{2+}\) than those in control cells (Fig. 2A). To exclude that the induction of baseline Ca\(^{2+}\) oscillations may have been due to this increasing Ca\(^{2+}\)-removing capacity from the cytosol, we have as a control overexpressed SERCA1 in COS-1 cells. In the absence of thapsigargin, permeabilized SERCA1-overexpressing and Pmr1-overexpressing cells accumulated about the same amount of Ca\(^{2+}\) in their respective Ca\(^{2+}\) stores (Fig. 2A).

Whereas Pmr1-overexpressing cells could easily be identified by their [Ca\(^{2+}\)] responses to ATP in the presence of thapsigargin, cells transfected with SERCA1 could not be detected in a similar manner. Therefore, immunocytochemical staining was performed on the same population of cells as used for the collection of the data on [Ca\(^{2+}\)] responses. Fig. 8C shows a typical immunocytochemical staining of a population of COS-1 cells exposed to the SERCA1 expression vector. About 50% of the cells overexpressed SERCA1.

In contrast to what was observed for Pmr1-overexpressing cells, baseline Ca\(^{2+}\) oscillations induced by 1 μM ATP occurred in only 3% of the total population of cells exposed to the SERCA1 transfection vector (Fig. 5C). These Ca\(^{2+}\) spikes quickly decreased both in frequency and amplitude. None of the SERCA1-overexpressing cells oscillated in response to 100 μM ATP (Fig. 8C). Overexpression of an intracellular Ca\(^{2+}\) pump per se was therefore not sufficient to set up baseline Ca\(^{2+}\) spiking.

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**Fig. 9.** Ca\(^{2+}\) signals induced by 0.2 μM ATP in control COS-1 cells. The cells were not pretreated with thapsigargin. The frequency of occurrence of the various responses is given (n = 1136). The arrow in panel A, representing the addition of 100 μM ATP, illustrates that the spikes had a low amplitude.

**Fig. 10.** Immunoblotting analysis of IP$_3$ receptor expression. Antibody detection of IP$_3$ receptor type 1 (IP3R1, A), type 2 (IP3R2, B) and type 3 (IP3R3, C), and of Pmr1 (D) and SERCA2b (E) in microsomes from non-transfected control COS-1 cells (Contr), from Pmr1-transfected COS-1 cells (PMR1), from SERCA2b-transfected COS-1 cells (SERCA), and from CHO cells (CHO). Each lane contained 75 μg of protein. Antibodies used were Rbt04 (1/2000, A), Rbt02 (1/500, B), MMAType3 (1/750, C), anti-Pmr1 (1/40,000, D) and anti-SERCA2b (1/10,000, E). Only the relevant parts of the blots are shown. Arrows indicate the position of the protein under investigation. Blots are typical for four experiments.
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IP₃ Receptor Expression in COS-1 Cells—To exclude that the induction of baseline Ca²⁺ oscillations in Pmr1-containing cells would have been due to possible effects of the presence of this pump on the level of IP₃ receptor expression, we have measured the levels of the three IP₃ receptor isoforms in microsomes of control, of Pmr1-overexpressing and of SERCA-overexpressing COS-1 cells. The levels of the three isoforms were not affected by the overexpression of Pmr1 or SERCA (Fig. 10, A–C). Neither was the endogenous level of SERCA2b affected by the overexpression of Pmr1 (Fig. 10E).

Ca²⁺ Oscillations in Thapsigargin-pretreated HeLa Cells—HeLa cells were pretreated with 2 µM thapsigargin to eliminate the endoplasmic reticulum as a functional Ca²⁺ store and stimulated with 100 µM histamine. About 86% of the cells maintained a stable [Ca²⁺]i (Fig. 11A), and 14% responded with abortive Ca²⁺ spikes (Fig. 11B and C). These oscillations had a remarkably long latency, which was often in the range of 30 to 60 min. These oscillations depended on the presence of histamine and disappeared when histamine was removed. Interestingly, removal of the agonist often resulted in a transient increase in the amplitude (Fig. 11B) or the frequency of the spikes (Fig. 11C), after which the [Ca²⁺]i returned to its pre-stimulation level. Ilimaquinone stopped the histamine-induced Ca²⁺ oscillations (Fig. 11D).

DISCUSSION

Cytosolic Ca²⁺ oscillations are due to cycles of release and subsequent re-uptake of internally stored Ca²⁺. The experimental data we have obtained fully support the hypothesis that baseline Ca²⁺ oscillations can be initiated by a SERCA-independent, probably Pmr1-replenished Ca²⁺ store. In COS-1 cells, the endogenous Ca²⁺-storage capacity of non-mitochondrial organelles other than the endoplasmic reticulum was small, because, in the presence of the SERCA-specific inhibitor thapsigargin, permeabilized COS-1 cells failed to accumulate Ca²⁺ at detectable levels. They did so, however, after transfection with Pmr1 (Fig. 2A) (12). In agreement with these observations, intact non-transfected COS-1 cells did not produce changes in [Ca²⁺]i, when stimulated with ATP in the presence of thapsigargin, whereas Pmr1-overexpressing cells were responsive. These cells thus made possible the study specifically of the effect of overexpression of the Pmr1 Ca²⁺ pump of the Golgi on the setting up of intracellular Ca²⁺ signals.

ATP-stimulated COS-1 cells that overexpressed the Pmr1 Golgi Ca²⁺ pump produced very stable baseline Ca²⁺ spikes, even after incubation in thapsigargin to inhibit the SERCA Ca²⁺ pumps in the endoplasmic reticulum. Such stable oscillatory responses never occurred in control cells with a functioning endogenous Ca²⁺ pump in the endoplasmic reticulum or in cells overexpressing the reticulum-targeted SERCA1 Ca²⁺ pump. These oscillations had all the typical characteristics of baseline cytosolic Ca²⁺ oscillations: (i) The spikes had a cell-specific shape; (ii) they could be abortive; (iii) the first [Ca²⁺]i rise was often preceded by a long latency; (iv) the frequency of the spikes increased when the [ATP] was increased; (v) Ca²⁺ was released from an internal Ca²⁺ store, because the oscillation occurred for a limited period of time in Ca²⁺-free solution; and (vi) the oscillation needed extracellular Ca²⁺ to be sustained.

A fast release of Ca²⁺ after a period of latency, reminiscent of that occurring during Ca²⁺ oscillations and similarly induced by Pmr1 overexpression, was also observed in the continuous presence of 100 µM ATP when the extracellular [Ca²⁺]i was suddenly raised from 0 to 1.5 mM (Fig. 3C). In control (Fig. 3, A and B) and SERCA1-overexpressing COS-1 cells (Fig. 3D) the rise of [Ca²⁺]i was much more gradual and not preceded by a period of latency. The fast time course of the increase of [Ca²⁺]i induced by Pmr1 overexpression strongly suggests a regenerative nature of the phenomenon and is therefore suggestive for an “all-or-none” release from an intracellular Ca²⁺ store, occurring when a threshold level of store filling has been exceeded. In agreement with this hypothesis, there always was a long latency period between the re-addition of Ca²⁺ and the fast rise of [Ca²⁺]i in Pmr1-overexpressing cells. Although it cannot be excluded that the delay was caused by a slow activation of capacitative Ca²⁺ entry, it is more likely that Ca²⁺ entry induced by the Ca²⁺-depleted endoplasmic reticulum (39) started immediately after addition of Ca²⁺. This incoming Ca²⁺ would then charge the IP₃-sensitive Pmr1-containing Ca²⁺ store, where the [IP₃] rise might be insufficient to open the Ca²⁺ channel directly due to the lower IP₃ sensitivity of this compartment. The loading of this pool then sensitizes it to the ambient level of IP₃ until a critical Ca²⁺ content is reached and a Ca²⁺ spike is triggered. This sensitization of the IP₃
Ca\(^{2+}\) Spiking in Pmr1-overexpressing COS-1 Cells

receptor may be due to the increasing luminal [Ca\(^{2+}\)] (20, 30) or the small rise in [Ca\(^{2+}\)], once this compartment became overloaded (40–43). The transfer of Ca\(^{2+}\) from its entry site at the plasma membrane into the Pmr1-containing Ca\(^{2+}\) store was not accompanied by a global rise in [Ca\(^{2+}\)]. A similar situation has been reported in pancreatic acinar cells, where recharging of the intracellular Ca\(^{2+}\) stores in the secretory pool with Ca\(^{2+}\) entering the cell at the basal pool also occurred without a rise in [Ca\(^{2+}\)] (44). Mn\(^{2+}\)-quenching experiments of cytoplasmic Ca\(^{2+}\)-sensitive dyes (data not shown) could not discriminate between delayed and immediate Ca\(^{2+}\) entry, possibly because Mn\(^{2+}\) is also rapidly transported by Pmr1 (12, 45).

COS-1 cells express mainly the type-3 (about 60%) and the type-2 IP\(_3\) receptor (about 30%), with only low levels of the type-1 IP\(_3\) receptor (46). We have found direct ATP binding to recombinant type-1 and type-3 IP\(_3\) receptors, which is in agreement with a stimulatory effect on IP\(_3\)-induced Ca\(^{2+}\) release in cell types mainly expressing these IP\(_3\) receptor isoforms (47). A particular characteristic of the type-2 IP\(_3\) receptor is its insensitivity to ATP (48). Genetically engineered B cells expressing both the type-1 and -2 IP\(_3\) receptor or expressing both the type-2 and -3 IP\(_3\) receptor indeed lost the prominent ATP dependence that is characteristic of the type-1 IP\(_3\) receptor and to a lesser extent of the type-3 IP\(_3\) receptor (48). The lack of ATP sensitivity of the IP\(_3\)-induced Ca\(^{2+}\) release from the Pmr1-containing compartment in COS-1 cells would be compatible with the presence of the type-2 IP\(_3\) receptor in this compartment. Available antibodies against the type-2 IP\(_3\) receptor are, however, of insufficient quality to directly immunolocalize with certainty this IP\(_3\) receptor isoform. Alternatively, the possibility remains that another IP\(_3\) receptor isoform is present in this compartment but has lost its responsiveness to ATP, due to the presence of other regulatory elements.

What is the nature of the Pmr1-expressing Ca\(^{2+}\) store? Pmr1 is a Golgi-targeted Ca\(^{2+}\) pump. Immunocytochemistry reveals that Pmr1 is present in the region that stains positive for the Golgi-specific 58K protein (Fig. 1A). However, weak staining was also present outside the Golgi apparatus. There are two possible explanations for this discrepancy. Either Pmr1 is only present in the Golgi compartment, in which case the Golgi marker would be unable to stain the whole Golgi compartment, or the Pmr1 antibody would in addition stain other proteins than Pmr1. Alternatively, some of the Pmr1 immunoreactivity is present outside the Golgi compartment. These proteins could be non-functional Ca\(^{2+}\) pumps being in the process of synthesis in the endoplasmic reticulum, or they can represent functional Pmr1 Ca\(^{2+}\) pumps that became incorporated in other subcellular compartments. It is unlikely that Pmr1 Ca\(^{2+}\) pumps present in the endoplasmic reticulum would have been responsible for the baseline Ca\(^{2+}\) spiking for the following reasons: (i) Control or SERCA1-overexpressing cells never processed the incoming Ca\(^{2+}\) during capacitative Ca\(^{2+}\) entry into a regenerative [Ca\(^{2+}\)], rise after a period of latency, whereas Pmr1-overexpressing cells always did. (ii) Control or SERCA1-overexpressing cells with a Ca\(^{2+}\)-loaded endoplasmic reticulum never produced baseline Ca\(^{2+}\) spikes, indicating that any overexpressed Pmr1 Ca\(^{2+}\) pumps that would have flowed into the endoplasmic reticulum could not have caused the induction of baseline Ca\(^{2+}\) oscillations in about half of the transfected cells. (iii) The functional properties of the Pmr1-containing Ca\(^{2+}\) store differed from those of the endoplasmic reticulum by the smaller inherent passive Ca\(^{2+}\) leak, the reduced sensitivity to IP\(_3\), and the insensitivity of their IP\(_3\) receptors to ATP. (iv) The Ca\(^{2+}\) oscillations were inhibited by ilimaquinone, which activates a heterotrimeric G protein in the Golgi apparatus, with the release of βγ subunits (49). βγ subunits bind to the pleckstrin homology domain of protein kinase D, which eventually causes vesiculation of Golgi stacks (50). The observed effects of ilimaquinone on Ca\(^{2+}\) signaling (Fig. 7) occurred within seconds, whereas fragmentation of the Golgi apparatus needed a much longer time. In addition, brefeldin A did not have the same effect on ATP-induced Ca\(^{2+}\) signaling as ilimaquinone (Fig. 7D). These findings indicate that the effect of ilimaquinone did, therefore, not depend on vesiculation of the Golgi complex. We propose that the state of phosphorylation of one of the substrates of protein kinase D may be critically involved in setting up the ATP-induced oscillation. All these findings make it very likely that the Ca\(^{2+}\) oscillations originate in the Golgi compartment.

Some of the functional properties that we observed for the Pmr1-containing compartment are in agreement with what has been reported for the Golgi apparatus in intact cells. The reduced passive Ca\(^{2+}\) leak of the Pmr1-containing Ca\(^{2+}\) store in permeabilized COS-1 cells corresponds with observations in LLC-PK\(_1\) epithelial cells, Swiss 3T3 fibroblasts, and L\(_5\) rat myoblasts, where the Golgi apparatus was substantially more resistant to Ca\(^{2+}\) depletion than the other regions of the cells (51). The difference in EC\(_{50}\) for IP\(_3\)-induced Ca\(^{2+}\) release (1.1 μM IP\(_3\) for the endoplasmic reticulum and 2.3 μM IP\(_3\) for the Pmr1-induced Ca\(^{2+}\) store) is in agreement with measurements of the luminal free [Ca\(^{2+}\)] within organelles in permeabilized BHK-21 cells, which showed a lower responsiveness to IP\(_3\) in the perinuclear Golgi region than in the other regions of the cell (29).

We have also investigated normal non-transfected HeLa cells, because the physiological relevance of findings obtained on cells overexpressing proteins could be questioned. When incubated in the presence of the SERCA-specific inhibitor thapsigargin, HeLa cells still managed to set up abortive Ca\(^{2+}\) spikes. The source of activator Ca\(^{2+}\) was therefore not the endoplasmic reticulum. An intracellular Ca\(^{2+}\) store filled by a non-SERCA type of Ca\(^{2+}\) pump could be involved. The Golgi complex seems to be a good candidate to fulfill this function in HeLa cells. First, active Ca\(^{2+}\) uptake in the latter compartment is mediated by a Pmr1-type of ion-transport ATPases, which is insensitive to thapsigargin and which is also expressed in HeLa cells (data now shown). Second, the Golgi apparatus in HeLa cells can function as a Ca\(^{2+}\) store that is able to release Ca\(^{2+}\) via IP\(_3\)-mediated pathways (10). Because of the lack of specific inhibitors of the Golgi Ca\(^{2+}\) pump, we have also here relied on ilimaquinone. Ilimaquinone stopped the histamine-induced Ca\(^{2+}\) oscillations in HeLa cells with functionally depleted endoplasmic reticulum Ca\(^{2+}\) stores. These observations on untransfected cells suggest that the Golgi complex may also play a role in cells where Pmr1 was not overexpressed. There are indeed early studies directly localizing Ca\(^{2+}\) to a Golgi-like structure in intact normal cells (51). Specific inhibitors of Pmr1 will be needed to further explore the role of the Golgi apparatus.

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