Ca\(^{2+}\) Uptake and Release Properties of a Thapsigargin-insensitive Nonmitochondrial Ca\(^{2+}\) Store in A7r5 and 16HBE14o– Cells*

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In a previous study we overexpressed the thapsigargin (tg)-insensitive Pmr1 Ca\(^{2+}\) pump of the Golgi apparatus of Caenorhabditis elegans in COS-1 cells and studied the properties of the Ca\(^{2+}\) store into which it was integrated. Here we assessed the properties of an endogenous tg-insensitive nonmitochondrial Ca\(^{2+}\) store in A7r5 and 16HBE14o– cells, which express a mammalian homologue of Pmr1. The tg-insensitive Ca\(^{2+}\) store was considerably less leaky for Ca\(^{2+}\) than the sarco(endo)plasmic-reticulum Ca\(^{2+}\)-ATPase (SERCA)-containing Ca\(^{2+}\) store. Moreover like for the worm Pmr1 Ca\(^{2+}\) pump expressed in COS-1 cells, Ca\(^{2+}\) accumulation into the endogenous tg-insensitive store showed a 2 orders of magnitude lower sensitivity to cyclopiazonic acid than the SERCA-mediated transport. 2,5-Di-(tert-butyl)-1,4-benzoxydroquinone was only a very weak inhibitor of the tg-insensitive Ca\(^{2+}\) uptake in A7r5 and 16HBE14o– cells and in COS-1 cells overexpressing the worm Pmr1. Inositol 1,4,5-trisphosphate released 11% of the Ca\(^{2+}\) accumulated in permeabilized A7r5 cells pretreated with tg with an EC\(_{50}\) that was 5 times higher than for the SERCA-containing Ca\(^{2+}\) store but failed to release Ca\(^{2+}\) in 16HBE14o– cells. In the presence of tg, 15% of intact A7r5 cells responded to 10 \(\mu\)M arginine-vasopressin with a small rise in cytosolic Ca\(^{2+}\) concentration after a long latency. In conclusion, A7r5 and 16HBE14o– cells express a Pmr1-containing Ca\(^{2+}\) store with properties that differ substantially from the SERCA-containing Ca\(^{2+}\) store.

Many cells use inositol 1,4,5-trisphosphate (IP\(_{3}\)) as a second messenger to generate intracellular Ca\(^{2+}\) signals (1). IP\(_{3}\) binds to the IP\(_{3}\) receptor, a Ca\(^{2+}\) channel found in the endoplasmic reticulum (ER). Ca\(^{2+}\) uptake into the ER, which represents the large tg-insensitive Ca\(^{2+}\) store observed in those cells was 33% responsive to IP\(_{3}\), albeit with a 3 times lower sensitivity than the tg-sensitive Ca\(^{2+}\) store (14). The heterologous expression of a Pmr1 Ca\(^{2+}\) pump belonging to the Pmr1 family of Ca\(^{2+}\) transport ATPases differing from the SERCA Ca\(^{2+}\) pumps by their insensitivity to tg has been reported in the Golgi apparatus (7, 11–13).

Recently we expressed the Caenorhabditis elegans Pmr1 Ca\(^{2+}\) pump in COS-1 cells and studied the functional properties of the Ca\(^{2+}\) store to which it was targeted (7, 14). The most important results of these studies were that Pmr1 largely co-localized with the Golgi apparatus and that the large tg-insensitive Ca\(^{2+}\) store observed in those cells was 53% responsive to IP\(_{3}\), albeit with a 3 times lower sensitivity than the tg-sensitive Ca\(^{2+}\) store (14). The heterologous expression of a Pmr1 Ca\(^{2+}\) pump from a different species, however, did not allow us to draw conclusions about the significance of endogenous Ca\(^{2+}\) signaling by the Golgi compartment in normal cells. We therefore screened for the presence of a Ca\(^{2+}\) store with similar properties as the Pmr1-induced Ca\(^{2+}\) store in nontransfected cells. Here we report on the properties of such a store in rat aortic A7r5 smooth muscle cells and in 16HBE14o– human bronchial mucosal cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**A7r5, COS-1, and 16HBE14o– cells were cultured as described previously (7, 15, 16). For \(\text{Ca}^{2+}\) fluxes cells were seeded in 12-well dishes (4 cm\(^2\); Costar, Cambridge, MA) at a density of \(-10^4\) cells cm\(^{-2}\), and for Ca\(^{2+}\) imaging experiments cells were seeded in Coverglass Chambers (Nunc Inc., Naperville, IL) at a density of \(5 \times 10^4\) cells cm\(^{-2}\). Four days after plating, COS-1 cells were tran-

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1 The abbreviations used are: IP\(_{3}\), inositol 1,4,5-trisphosphate; tg, thapsigargin; [X], concentration of X; [Ca\(^{2+}\)], free cytosolic Ca\(^{2+}\) concentration; ER, endoplasmic reticulum; SERCA, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase; RT, reverse transcriptase.

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FIG. 1. Demonstration of a tg-insensitive nonmitochondrial Ca\(^{2+}\) store in A7r5 cells and 16HBE14o- cells. The nonmitochondrial Ca\(^{2+}\) stores of permeabilized A7r5 (A) and 16HBE14o- cells (B) were loaded for 45 min in the absence (C, dotted line) or presence of 10 \(\mu\)M tg (G, full line) and from time 0 onward were incubated in a Ca\(^{2+}\)-free efflux medium, and their Ca\(^{2+}\) contents were plotted as a function of time. Means \(\pm\) S.E. are shown for four experiments. S.E. values smaller than the individual data points are not shown. Passive binding of \(^{45}\)Ca\(^{2+}\) to the stores, i.e. the \(^{45}\)Ca\(^{2+}\) bound to the cells after loading in the presence of 10 \(\mu\)M A23187, was subtracted from both traces. Arrows a represent the tg-insensitive Ca\(^{2+}\) uptake, and arrows b represent the tg-sensitive part.

FIG. 2. Passive Ca\(^{2+}\) leak from the tg-sensitive and tg-insensitive nonmitochondrial Ca\(^{2+}\) store in A7r5 and 16HBE14o- cells. The nonmitochondrial Ca\(^{2+}\) stores of permeabilized A7r5 (A) and 16HBE14o- cells (B) were loaded with 4\(^{45}\)Ca\(^{2+}\) for 45 min and from time 0 onward were incubated in a Ca\(^{2+}\)-free efflux medium, and their Ca\(^{2+}\) contents were plotted as a function of time. SERCA-mediated uptake of Ca\(^{2+}\) (C, dotted line) was taken as the Ca\(^{2+}\) uptake in the absence of tg minus that in the presence of 10 \(\mu\)M tg (arrows b in Fig. 1). Ca\(^{2+}\) uptake by the tg-insensitive Ca\(^{2+}\) store (G, full line) was measured in a medium containing 10 \(\mu\)M tg as the difference in Ca\(^{2+}\) uptake in the presence and absence of 10 \(\mu\)M A23187 (arrows a in Fig. 1). Means \(\pm\) S.E. are shown for four experiments. S.E. values smaller than the individual data points are not shown.

RESULTS AND DISCUSSION

Demonstration of an Endogenous Tg-insensitive Nonmitochondrial Ca\(^{2+}\) Store in A7r5 and 16HBE14o- Cells—All members of the SERCA family of Ca\(^{2+}\) pumps are irreversibly inhibited by tg with similar affinity (2). We loaded the Ca\(^{2+}\) stores of permeabilized A7r5 (Fig. 1A) and 16HBE14o- cells (Fig. 1B) in the absence (open symbols and dotted line) or presence (closed symbols and full line) of a supramaximal concentration of tg (10 \(\mu\)M) and then investigated how the Ca\(^{2+}\) contents of the respective Ca\(^{2+}\) stores decreased as a function of time of incubation in efflux medium. A subcompartment of the Ca\(^{2+}\) stores could still actively sequester Ca\(^{2+}\) despite the presence of high tg levels to block SERCA Ca\(^{2+}\) pumps. Of the total Ca\(^{2+}\) uptake in A7r5 cells, 92% involved a tg-sensitive SERCA Ca\(^{2+}\) pump (arrow b in Fig. 1A), and 8% was mediated by a tg-insensitive Ca\(^{2+}\) uptake mechanism (arrow a in Fig. 2A).
and absence of $10^{-6}$ M NaN$_3$, $10^{-6}$ M oligomycin, and $10^{-6}$ M antimycin A and could therefore not account for the tg-resistant fraction.

**Passive Ca$^{2+}$ Leak from the Tg-insensitive Nonmitochondrial Ca$^{2+}$ Store in A7r5 and 16HBE140− Cells**—After loading the stores to steady state, their passive permeability to Ca$^{2+}$ was assessed by switching to an efflux medium containing $2^{-4}$ M EGTA with no added Ca$^{2+}$ or ATP. The Ca$^{2+}$ efflux that occurred under these conditions can be considered as unidirectional since the calculated free [Ca$^{2+}$] in the efflux medium ($<10^{-6}$ M) was below the threshold to stimulate the Ca$^{2+}$ pumps, and no ATP was present to fuel the pumps. Fig. 2 shows for A7r5 (Fig. 2A) and 16HBE140− cells (Fig. 2B) the decrease in Ca$^{2+}$ content as a function of time for both the tg-insensitive compartment (closed circles, full line) and the SERCA-containing Ca$^{2+}$ store (open circles, dotted line). It is clear that the rates of Ca$^{2+}$ loss from the tg-insensitive compartment in A7r5 cells and especially in 16HBE140− cells were significantly smaller than those of the SERCA-containing Ca$^{2+}$ store. These differences were not a consequence of the different initial Ca$^{2+}$ content of the two stores since the initial level of store loading has no effect on the passive Ca$^{2+}$ leak (18). The data in Fig. 2

**FIG. 3.** RT-PCR of Pmr1 in A7r5 and 16HBE140− cells. Gel electrophoresis of RT-PCR products using primers specific for the rat (A7r5 cells) and the human Pmr1 sequence (16HBE140− cells (HBE)). Single bands of the predicted length were amplified. The gel was stained with Vistra Green. M, molecular marker.

**FIG. 4.** Effect of cyclopiazonic acid on Ca$^{2+}$ uptake by the tg-sensitive and tg-insensitive Ca$^{2+}$ stores in permeabilized A7r5 and 16HBE140− cells and on Ca$^{2+}$ uptake mediated by SERCA or Pmr1 in permeabilized COS-1 cells. The nonmitochondrial Ca$^{2+}$ stores of permeabilized A7r5 (A), 16HBE140− (B), and COS-1 cells (C) were loaded for 45 min in the presence of the indicated cyclopiazonic acid. The Ca$^{2+}$ uptake in the presence of the inhibitor expressed as a percentage of that in its absence (means ± S.E., $n = 4$) is plotted as a function of the cyclopiazonic acid. The tg-sensitive Ca$^{2+}$ uptake in A7r5, 16HBE140−, and nontransfected COS-1 cells mediated by SERCA (C, dotted line) was taken as the Ca$^{2+}$ uptake in the absence of tg minus that in the presence of $10^{-6}$ M tg (arrows b in Fig. 1). Ca$^{2+}$ uptake by the tg-sensitive Ca$^{2+}$ store (●, full line) in A7r5 and 16HBE140− cells was measured in a medium containing $10^{-6}$ M tg as the difference in Ca$^{2+}$ uptake in the presence and absence of $10^{-6}$ M antimycin A23187 (arrows a in Fig. 1). Ca$^{2+}$ uptake by Pmr1 in COS-1 cells (●, full line) was taken as the difference in Ca$^{2+}$ uptake between Pmr1-overexpressing and control COS-1 cells in a medium containing $10^{-6}$ M tg. Cyclopiazonic acid was dissolved in dimethyl sulfoxide, the concentration of which was constant in all experiments (1%).

**FIG. 5.** Effect of 2,5-di-(tert-butyl)-1,4-benzoquinone on Ca$^{2+}$ uptake by the tg-sensitive and tg-insensitive Ca$^{2+}$ stores in permeabilized A7r5 and 16HBE140− cells and on Ca$^{2+}$ uptake mediated by SERCA or Pmr1 in permeabilized COS-1 cells. The nonmitochondrial Ca$^{2+}$ stores of permeabilized A7r5 (A), 16HBE140− (B), and COS-1 cells (C) were loaded for 45 min in the presence of the indicated (2,5-di-(tert-butyl)-1,4-benzohydroquinone). The Ca$^{2+}$ uptake in the presence of the inhibitor expressed as a percentage of that in its absence (means ± S.E., $n = 4$) is plotted as a function of the inhibitor concentration. The tg-sensitive Ca$^{2+}$ uptake mediated by SERCA (C, dotted line) and the Ca$^{2+}$ uptake by the tg-insensitive Ca$^{2+}$ store in A7r5 and 16HBE140− cells and by Pmr1 in COS-1 cells (●, full line) were measured as described in the legend to Fig. 4. 2,5-Di-(tert-butyl)-1,4-benzoquinone was dissolved in dimethyl sulfoxide, the concentration of which was constant in all experiments (1%).
are in agreement with our earlier report that also the Pmr1-induced Ca\textsuperscript{2+} store in COS-1 cells was less leaky as compared with the ER in these cells (see 2B in Ref. 14).

A7r5 and 16HBE14o—Cells Express Pmr1—Pmr1 is a tg-sensitive Ca\textsuperscript{2+} pump present in the Golgi apparatus (7, 13), making it a likely candidate for the Ca\textsuperscript{2+} uptake mechanism in the presence of tg. Fig. 3 shows that Pmr1 could indeed be demonstrated in A7r5 and 16HBE14o—cells at the mRNA level. Another argument for the presence of Pmr1 is that the pharmacology of the Ca\textsuperscript{2+} uptake mechanism of the tg-insensitive Ca\textsuperscript{2+} store in A7r5 and 16HBE14o—cells and that of the overexpressed Pmr1 Ca\textsuperscript{2+} pump in COS-1 cells were similar as discussed in the following paragraphs.

Ca\textsuperscript{2+} uptake by the tg-insensitive nonmitochondrial Ca\textsuperscript{2+} store was inhibited by the mycotoxin cyclopiazonic acid with an IC\textsubscript{50} of 165 \textmu M in A7r5 cells (Fig. 4A, closed symbols and full line) and 337 \textmu M in 16HBE14o—cells (Fig. 4B, closed symbols and full line). These values were 2 orders of magnitude higher than the IC\textsubscript{50} value found to inhibit SERCA-mediated Ca\textsuperscript{2+} uptake in A7r5 cells (1.0 \textmu M, open circles and dotted line in Fig. 4A) and 16HBE14o—cells (1.6 \textmu M, open circles and dotted line in Fig. 4B). The inhibition curves for the tg-insensitive Ca\textsuperscript{2+} uptake were also steeper than those of SERCA. The IC\textsubscript{50} values for SERCA inhibition were higher than the previously reported value of 10 \textmu M in the uptake medium since ATP protects the enzyme in a competitive manner against inhibition by cyclopiazonic acid (19). Fig. 4C shows how cyclopiazonic acid affected exogenous Pmr1 in COS-1 cells overexpressing this Ca\textsuperscript{2+} pump (7, 14). In this assay system, Pmr1-induced Ca\textsuperscript{2+} pumping could be specifically measured as the difference in Ca\textsuperscript{2+} uptake between Pmr1-overexpressing COS-1 cells and control cells in a medium containing 10 \textmu M tg. The closed circles and full line in Fig. 4C illustrate that cyclopiazonic acid inhibited Pmr1 with an IC\textsubscript{50} of 294 \textmu M, while SERCA in these cells was half-maximally inhibited at 0.7 \textmu M (Fig. 4C, open circles and dotted line). Pmr1 was therefore 2 orders of magnitude less sensitive to cyclopiazonic acid than was SERCA. The inhibition curve for Pmr1 was also steeper than that of SERCA. It is evident that the values obtained for the exogenous Pmr1 Ca\textsuperscript{2+} pump in COS-1 cells are in excellent agreement with the values found for the endogenous tg-insensitive Ca\textsuperscript{2+} pump in A7r5 and 16HBE14o—cells.

2,5-Di-(tert-butyl)-1,4-benzoquinone, another inhibitor of the SERCA Ca\textsuperscript{2+} pumps (20), was only a very weak inhibitor of the tg-insensitive nonmitochondrial Ca\textsuperscript{2+} store since even very high concentrations (1 mM) only induced a partial inhibition in A7r5 cells (closed circles and full line in Fig. 5A), in 16HBE14o—cells (closed circles and full line in Fig. 5B), and of the overexpressed Pmr1 in COS-1 cells (closed circles and full line in Fig. 5C). The values obtained for the exogenous Pmr1 Ca\textsuperscript{2+} pump in COS-1 cells are therefore again in excellent agreement with the values found for the endogenous tg-insensitive Ca\textsuperscript{2+} pump in A7r5 and 16HBE14o—cells. In contrast, Ca\textsuperscript{2+} uptake mediated by SERCA was inhibited with an IC\textsubscript{50} of 1.4 \textmu M in A7r5 cells, 1.3 \textmu M in 16HBE14o—cells, and 1.0 \textmu M in COS-1 cells (open circles and dotted line, respectively, in Fig. 5, A, B, and C).

Ca\textsuperscript{2+} Release from the Tg-insensitive Nonmitochondrial Ca\textsuperscript{2+} Store in A7r5 and 16HBE14o—Cells—Permeabilized

![Figure 6](http://www.jbc.org/)

**Fig. 6.** Effect of IP\textsubscript{3} on the tg-insensitive Ca\textsuperscript{2+} store in A7r5 and 16HBE14o—cells. A and B, permeabilized A7r5 (A) and 16HBE14o—cells (B) were loaded to steady state with Ca\textsuperscript{2+} in the presence of 10 \textmu M tg and from time 0 onward were incubated in efflux medium. At the time indicated by the horizontal bar, 100 \textmu M IP\textsubscript{3} (•, full line) or 10 \textmu M A23187 (□, dashed line) were added for 2 min. Ca\textsuperscript{2+} release is plotted as fractional loss, i.e. the amount of Ca\textsuperscript{2+} released in 2 min divided by the total store Ca\textsuperscript{2+} content at that time. Means ± S.E. are shown for three experiments. C, [IP\textsubscript{3}] dependence of the Ca\textsuperscript{2+} release from the tg-sensitive (○, dotted line) and tg-insensitive Ca\textsuperscript{2+} store (•, full line) in A7r5 cells. The Ca\textsuperscript{2+} release is expressed as a percentage of that induced by 10 \textmu M A23187. The arrows point to the EC\textsubscript{50} values for IP\textsubscript{3}-induced Ca\textsuperscript{2+} release. Results from a typical experiment are shown (n = 3).

![Figure 7](http://www.jbc.org/)

**Fig. 7.** [Ca\textsuperscript{2+}], measurements in arginine-vasopressin-stimulated A7r5 cells. A, effect of 10 \textmu M arginine-vasopressin (black bar) on [Ca\textsuperscript{2+}], in an A7r5 cell. A similar response occurred in 100% of the cells (n = 210). B, effect of 10 \textmu M arginine-vasopressin on [Ca\textsuperscript{2+}], in a cell pretreated with 10 \textmu M tg. This response occurred in only 15% of the cells; the others failed to respond (n = 180).
A7r5 cells were loaded to steady state with Ca^{2+} in the presence of 10 μM tg and a mixture of mitochondrial inhibitors and then incubated in efflux medium and stimulated with 100 μM IP_3 (Fig. 6A, circles and full line) or 10 μM A23187 as Ca^{2+} ionophore (Fig. 6A, squares and dashed line). IP_3 induced a partial Ca^{2+} release from this compartment. Inositol 1,3,4,5-tetrakisphosphate (100 μM), cyclic ADP-ribose (100 μM), and caffeine (20 mM) all failed to release Ca^{2+} under these conditions (data not shown). Nicotinic acid adenine dinucleotide phosphate (100 μM), which has been reported to release Ca^{2+} from a tg-insensitive nonmitochondrial Ca^{2+} store in other cell types (21) and which was also reported to release Ca^{2+} in A7r5 cells (22), also was unable to release Ca^{2+} under these conditions (data not shown). In 16HEB140− cells, no significant release was observed upon addition of 100 μM IP_3 (Fig. 6B, circles and full line) or 100 μM inositol 1,3,4,5-tetrakisphosphate, 100 μM cyclic ADP-ribose, 20 mM caffeine, and 100 mM nicotinic acid adenine dinucleotide phosphate (data not shown).

To compare the properties of the IP_3 receptors in the tg-insensitive Ca^{2+} store with those in the SERCA-containing Ca^{2+} store in A7r5 cells, both types of stores were loaded with 45Ca^{2+} and then challenged with IP_3 in efflux medium. The open circles and dotted line in Fig. 6C illustrate the Ca^{2+} release from the SERCA-containing Ca^{2+} store as a function of the [IP_3]. The closed circles and full line are the values for the tg-insensitive Ca^{2+} store. The EC_{50} was 1.2 μM IP_3 for the SERCA-containing Ca^{2+} store (dotted arrow) and 5.2 μM IP_3 for the tg-insensitive Ca^{2+} store (solid arrow). A maximal [IP_3] released 83% of the ionophore-releasable Ca^{2+} from the SERCA-containing Ca^{2+} store but only released 11% from the tg-insensitive Ca^{2+} store. A similar incomplete Ca^{2+} release at the highest [IP_3] and a higher EC_{50} for IP_3 were previously also observed for the Pmr1-induced Ca^{2+} store in COS-1 cells (14).

Ca^{2+} Signals in Intact A7r5 Cells—The addition of 10 μM arginine-vasopressin to control A7r5 cells produced an immediate [Ca^{2+}], rise (Fig. 7A). This immediate response was abolished when the cells were pretreated with 10 μM tg (Fig. 7B). Under these conditions, a delayed abortive [Ca^{2+}], rise occurred in 15% of the cells. This finding indicates that the tg-insensitive Ca^{2+} store in some cells was large enough to be discharged by external stimulation of the cell. Interestingly the Ca^{2+} spikes in Pmr1-overexpressing COS-1 cells often also occur after a long latency (14). None of the 16HEB140− cells responded to 100 μM ATP in the presence of tg.

Conclusions—We demonstrated in A7r5 and 16HEB140− cells a tg-insensitive Ca^{2+} store that was less leaky for Ca^{2+} than the ER. Based on our findings that (i) Pmr1 was present in A7r5 and 16HEB140− cells and that (ii) the Ca^{2+} uptake mechanism of the tg-insensitive Ca^{2+} store in A7r5 and 16HEB140− cells and the overexpressed Pmr1 Ca^{2+} pump in COS-1 cells had the same sensitivity to cyclopiazonic acid and 2,5-di-(tert-butyl)-1,4-benzohydroquinone, we propose that the Pmr1 Ca^{2+} pump was responsible for loading up the tg-insensitive Ca^{2+} store in A7r5 and 16HEB140− cells. Since Pmr1 is expressed in the Golgi apparatus (11), this tg-insensitive Ca^{2+} store in A7r5 and 16HEB140− cells probably corresponds to the Golgi complex. IP_3 released 11% of the Ca^{2+} accumulated in this compartment in A7r5 cells with an EC_{50} that was 5 times higher than for the ER in these cells. This store could also be released in intact cells during agonist stimulation. Heterogeneous nonmitochondrial Ca^{2+} stores therefore exist in A7r5 and 16HEB140− cells.

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