The plasma membrane Ca\textsuperscript{2+}-ATPase pump (PMCA) is an integral component of the Ca\textsuperscript{2+} signaling system which participates in signal transduction during agonist stimulated cell activation. To better understand the physiological function of the pump, isoform 1a (PMCA1a) was over-expressed in rat aortic endothelial cells using a stable transfection system under the control of a cytomegalovirus promoter. The cell lines selected after transfection with PMCA1a construct, expressed 3-4-fold increased pump protein which was mostly targeted to the plasma membrane as indicated by immunoperoxidase staining. Ca\textsuperscript{2+} uptake assays in a membrane preparation indicated a 3-4-fold increase in Ca\textsuperscript{2+} pumping activity in the transfected cells, and the expressed PMCA1a showed typical dependence on Ca\textsuperscript{2+} and calmodulin for stimulation of activity. Measurement of [Ca\textsuperscript{2+}]\textsubscript{i} and [Ca\textsuperscript{2+}]\textsubscript{out} showed that expression of PMCA1a had a profound effect on different aspects of the Ca\textsuperscript{2+} signal. The peak increase in [Ca\textsuperscript{2+}]\textsubscript{i} evoked by ATP and/or thapsigargin was lower but the plateau phase was similar in the PMCA1a expressing cells. Accordingly, titration with ionomycin of Ca\textsuperscript{2+} content of internal stores, measurement of Ca\textsuperscript{2+} uptake into the thapsigargin- and oxalate-sensitive pool (endoplasmic reticulum) of isolated microsomes, Ca\textsuperscript{2+} uptake into streptolysin O-permeabilized cells, and analysis of SERCA mRNA and protein, showed that expression and activity of the SERCA pump was down-regulated in cells expressing PMCA1a pump. Expression of PMCA1a also down-regulated expression of the inositol 1,4,5-trisphosphate (IP\textsubscript{3})-activated Ca\textsuperscript{2+} channel and the rate of IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release in permeable cells, without affecting the affinity of the channel for IP\textsubscript{3}. On the other hand the rate of store depletion-dependent Ca\textsuperscript{2+} and Mn\textsuperscript{2+} influx entry into PMCA1a expressing cells was increased by about 2.6-fold. These changes prevented estimating the rate of pump-mediated Ca\textsuperscript{2+} efflux from changes in [Ca\textsuperscript{2+}]. Measurement of [Ca\textsuperscript{2+}]\textsubscript{out} showed that the rate of Ca\textsuperscript{2+} efflux in cells expressing PMCA1a was about 1.45-fold higher than Neo controls, despite the 4-fold increase in the amount of functional pump protein. The overall study points to the flexibility, interdependence, and adaptability of the different components of the Ca\textsuperscript{2+} signaling systems to regulate the expression and activity of each component and maintain a nearly constant Ca\textsuperscript{2+} signal.

The plasma membrane Ca\textsuperscript{2+} pump (PMCA)\textsuperscript{1} is the sole high affinity Ca\textsuperscript{2+} extrusion mechanism in the plasma membrane (1). In non-muscle cells, the pump plays a prominent role in Ca\textsuperscript{2+} extrusion than other mechanisms such as the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (2-7). PMCA activity is believed to be activated during hormonal stimulation of cells (2) and the increased Ca\textsuperscript{2+} efflux is concomitant with an enhanced phosphorylation of the Ca\textsuperscript{2+} pump protein (8, 9).

cDNA cloning indicates the presence of at least four major isoforms for the PMCA, which are encoded by distinct genes numbered “1 to 4” (1, 10–12). The primary transcripts of each gene may be alternatively spliced at their 3’ ends in a different way, further increasing the variety of pumps as denoted by letters “a to d” (1). While these isozymes show distinct tissue and cellular distribution, isoform 1b (PMCA1b) appears to be the major isoform that is expressed in all tissues examined (1, 13). Recent attempts to over-express the human gene 4 in COS cells have demonstrated the usefulness of transient transfection in relating the structure of the pump to its biochemical activity (14–17). However, a transient expression system does not allow the study of intracellular Ca\textsuperscript{2+} signaling in a metabolically active cell type, such as endothelial cells, due to the low transfection efficiency (normally 15% or less of the cells were transfected by the transient transfection system).\textsuperscript{2} In this report we focus on the stable transfection of the PMCA1a isoform of the pump in rat aortic endothelial cells (RAECs), which endogenously contain a low level of PMCA1b as the predominant species, while isoform 1a is either absent or undetectable by reverse transcriptase-polymerase chain reaction (13). This over-expression system allowed us to study the physiological function of PMCA during agonist stimulation. In addition, since no information is available regarding how over-expression of one calcium transporter may influence the expression of other calcium transporters, we have examined the effect of PMCA1a over-expression on the four major Ca\textsuperscript{2+} transport pathways responsible for cellular Ca\textsuperscript{2+} homeostasis, which includes intracellular pump (SERCA), the IP\textsubscript{3}-activated Ca\textsuperscript{2+} channel, the plasma membrane calcium entry pathway, and the PMCA (18, 19). The overall findings demonstrate the flexibility and coordinated regulation of all pathways, possibly on the gene level, to maintain constant Ca\textsuperscript{2+} signaling.

\section*{MATERIALS AND METHODS}

\textbf{Plasmid Construction—}The cDNA encoding rat plasma membrane Ca\textsuperscript{2+}-ATPase isoform 1a (PMCA1a) was excised from plasmid RB11-1.

\textsuperscript{1}The abbreviations used are: PMCA, plasma membrane Ca\textsuperscript{2+}-ATPase; RAEC, rat aortic endothelial cell; SERCA, sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase; ER, endoplasmic reticulum; IP\textsubscript{3}, inositol 1,4,5-trisphosphate; IP\textsubscript{3}R, IP\textsubscript{3} receptor; CRAC, Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} influx; Tg, thapsigargin; [Ca\textsuperscript{2+}]\textsubscript{i}, free intracellular Ca\textsuperscript{2+} concentration; [Ca\textsuperscript{2+}]\textsubscript{out}, free extracellular Ca\textsuperscript{2+} concentration; SLO, streptolysin O toxin; CHO, Chinese hamster ovary.

\textsuperscript{2}B.-F. Liu, X. Xu, R. Fridman, S. Muallem, and T. H. Kuo, unpublished data.
(10) (a gift from Dr. Gary Shull) with Bcl and BamHI, cloned into the eukaryotic expression vector pCDNA/NEO (Invitrogen) in their BamHI site. The resulting construct, named PMCA1a, construct and the vector control (without the cDNA insert) were used to transfected rat aortic endothelial cells separately.

Cell Culture and Stable Transfection—RAEC were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 25 μg/ml gentamicin as described previously (9). To establish cell lines that stably express rat PMCA1a, 6 μg/ml PMCA construct or vector alone was transfected into RAEC by the Lipofectin method (Life Technologies, Inc.). The neomycin-resistant colonies were selected with 90 μg/ml G418 in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Colonies were picked and grown as individual cell lines in the presence of 90 μg/ml G418. Each cell line was assayed by Western blot to confirm PMCA expression.

Northern Blot Analysis—Total RNA was isolated from cells in logarithmic growth phase, and samples were used for Northern analysis as described previously (9). After electrophoresis and transfer, the blots were hybridized with specific cDNA probes. The SERCA cDNA probe was a 330-base pair EcoRI/PstI fragment (nucleotides 892-1222) of the SERCA2 gene (20). This probe can recognize both SERCA isofoms 2 and 3 present in the RAEC. In the case of Fig. 6, a full-length SERCA3 cDNA (21) was used as a probe to detect SERCA3 mRNA.

Western Blot Analysis—The protein samples (cell lysate) were separated on 7% polyacrylamide gels that contained 0.1% SDS and transferred onto nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBS-Tween, then incubated with primary antibodies against PMCA1a as well as endogenous pump detected by using the alkaline phosphatase-conjugation method (9).

Immunoperoxidase Staining—The cells were grown on coverslips for immunostaining by avidin-biotin technique using a kit from Vector Laboratory. Samples on coverslips were fixed in acetone and then treated with monoclonal antibody 5F10 (Affinity BioReagents) against the human erythrocyte PMCA was described earlier (22). This antibody recognizes several plasma membrane calcium pump isoforms including PMCA1a in various tissues (13). The nitrocellulose membrane was treated with 5F10 and the expressed PMCA1a as well as endogenous pump detected by using the alkaline phosphatase-conjugation method (9).

Membrane Preparation—A crude membrane containing both plasma membrane and microsomes was prepared according to the procedure of Enyedi et al. (15). The cells were homogenized in a hypotonic solution of 10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 2 mM diethyldithioctetraoxane. The homogenate was diluted with an equal volume of 0.5 M sucrose, 0.3 M KCl, 2 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5, 10 mM EDTA to a protein concentration of 200 μg/ml. The suspension was centrifuged at 30,000 × g for 15 min. The supernatant was made up to 0.6 M KCl in KCl and 1.5 mM in EDTA (to remove calmodulin). The suspension was centrifuged at 100,000 × g for 40 min. The final pellet was resuspended in a solution containing 0.25 M sucrose, 0.25 M KCl, 10 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, and 20 mM CaCl2.

Ca2+/Calmodulin-dependent uptake into the microsomes was measured similar to the procedures of Ref. 15. However, a major difference being that under our conditions, it was not necessary to add 40 mM phosphate to improve the signal to noise ratio. Membrane vesicles (6 μg protein) were incubated at 37°C in 140 μl of medium containing 100 mM KCl, 20 mM Hepes, pH 7.4, 7 mM MgCl2, 5 mM Na2ATP, 4 μg/ml oligomycin, 0.5 mM ouabain, 100 μM CaCl2, labeled with 45Ca, and sufficient EGTA to obtain the desired free [Ca2+]. Thapsigargin (200 nM) was also included to inhibit the activity of the SERCA pumps. The reaction was started by the addition of 6 μM ATP. The incubation time was 5 min. The radioactive Ca2+ incorporation at each time point was determined by filtration (23). The transport activity was determined in the presence and absence of 240 mM calmidazolium. SERCA activity assay is based on the measurement of ATP-mediated, oxalate-dependent calcium uptake into microsomes according to Lytton et al. (24).

Measurements of Free Cytosolic 
Ca2+—[Ca2+]i was measured by image acquisition and analysis using the Meridian ACAS 570 or the PTI system described before (25). RAEC grown or attached to coverslips were loaded with Indo-1 or Fura 2 by incubation in loading buffer containing (in mM) 5.4 KCl, 137 NaCl, 0.44 KH2PO4, 4.2 NaHCO3, 0.34 Na2HPO4, 11.1 l-glucose, 5 Hepes, pH 7.3, 2 CaCl2, 0.1% bovine serum albumin. After 60 min incubation with Indo 1/AM or 30 min incubation with Fura 2/AM, the cells were rinsed, the coverslips were placed in a holder and mounted on the laser cyrometer equipped with an inverted microscope (Nikon i) and a stage of a Nikon inverted microscope (Fura 2). Calcium was released at an excitation wavelength of 360 nm and emission wavelengths of 405 and 485 nm. Fura 2 fluorescence was measured at excitation wavelengths of 355 and 380 nm. For measurement of [Ca2+]i, in cell suspension, Fura 2-loaded cells were suspended in a solution containing (in mM) 140 NaCl; 5 KCl; 1 MgCl2; 10 Hepes (pH 7.4 with NaOH); 10 glucose (solution A) and with or without 1 μM IP3. The cells were suspended in a buffer containing NaCl, and the cells were continuously stirred. Fluorescence was recorded at excitation wavelengths of 340 and 360 nm and an emission wavelength of 500 nm. When appropriate, the signals were calibrated as described below (26).

Measurement of [Ca2+]i—Cells were released from culture plates, washed twice in solution A, and kept on ice until use. Before each measurement about 4 × 106 cells were washed once with Ca2+-free solution A containing 0.2 mM MgCl2, and once with the same solution that was treated with Chelex 100 (Sigma). The cells were resuspended in 0.5 ml of a warm (37°C) Chelex-treated solution that contained 1 μM Fluo 3 and placed in a thermostated fluorimeter cuvette. EGTA in increments of 0.2 μM was added until medium [Ca2+]i was reduced to about 100 nM. When stimulated with a mixture of (final concentrations) 100 μM ATP and 2 μM Tg, Fluo 3 fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. The fluorescence of the dye present in the external medium was calibrated by addition of 1 mM CaCl2 to the incubation medium to obtain Fmax and then 10 μM EGTA and 20 mM NaOH to obtain Fmin. [Ca2+]i was calculated as described before using a Kd of 370 mM (2).

Measurement of Ca2+ Uptake and Release in Permeable Cells—Between 5 × 105 and 106 RAEC cells in solution A were washed twice with a solution containing 145 mM KCl and 10 mM Hepes (pH 7.4 with NaOH) and once with the same solution treated with Chelex 100. The cells were re-suspended in 0.5 ml of a warm, Chelex-treated solution containing 1 μM Fluo 3, 3 μM ATP, 5 mM MgCl2, 10 mM creatine phosphate, 5 units/ml creatine kinase, 10 μg/ml oligomycin, 10 μg/ml antimycin A, and 3 mg/ml streptolysin O (SLO) reagent (Murex). The cells were allowed to reduce medium [Ca2+]i using Ca2+ uptake into the Tg-sensitive stores. After stabilization of [Ca2+]i, IP3-mediated Ca2+ release was measured by addition of increasing concentrations of IP3 to the cuvette. The Ca2+ signal was calibrated as described above of 300.

Calculation and Statistics—All measurements of initial rates reported were derived from the maximal slope of a given curve of changes in [Ca2+]i, or [Ca2+]i. When appropriate, results are reported as mean ± S.E. of the indicated number of experiments. Statistical analysis was by Student’s t test. p < 0.05 and below was considered statistically significant.

RESULTS

Over-expression of Rat PMCA Isoform 1a in RAEC—Following G418 selection, 36 neomycin-resistant clones were isolated from the culture transfected with the PMCA1a construct. Screening by Western blot with the monoclonal antibody 5F10 (Fig. 1A) indicated varying degrees of PMCA1a over-expression (from 1-4 fold increase over the level of the native PMCA1b). Cells that were transfected with the vector alone (lane b) contained mainly the PMCA1b isoform and showed low amounts of PMCA1a protein, similar to the nontransfected parental RAEC (lane a). Fig. 1A shows only the screening results of six clones. After complete screening of all 36 clones, three clones that expressed approximately 3-4 fold higher PMCA1a protein, i.e. clones 8, 33 (Fig. 1A), and 35 (not shown), were selected for further study. In a different study using transient expression of PMCA1a construct in BSC-1 cells, we have established that the over-expressed protein has a molecular mass of 130 kDa (Fig. 1B, lower band) which matches the theoretical size based on the cDNA sequence of isoform 1a (10), while the major endogenous protein had a molecular mass of 135 kDa corresponding to isoform 1b (Fig. 1B, upper band). Over-expression of isoform 1a in RAEC was further confirmed
Ca\(^{2+}\) Pump Over-expression and Ca\(^{2+}\) Homeostasis

The Expressed PMCA1a is Functional and Is Largely Targeted to the Plasma Membrane—Fig. 2 shows the immunoperoxidase staining of the PMCA1a over-expressing clone as compared to the control cells transfected with the vector alone. The use of PMCA antibody 5F10 showed that higher levels of Ca\(^{2+}\) pump were expressed in the PMCA-transfected cells (clone 8, Fig. 2A) as compared with control cells (Fig. 2B). A pattern of a punctate, peripheral distribution of stained material was quite obvious in the clone 8 over-expressing PMCA1a (Fig. 2A) (observed as reddish-brown spots in a blue hematoxylin background), suggesting that at least a major portion of the over-expressed protein was associated with the plasma membrane. An additional observation in Fig. 2A in support of plasma membrane localization is the heaviest staining of the cytoplasmic perimeter of the cell where the superficial and underlying plasma membrane come to join. Since the distribution of the label on the cytoplasmic and/or nuclear surface is evenly scattered, it is unlikely that they represent ER localization. In control cells (Fig. 2B) minimal staining of endogenous proteins are detected due to its low abundance in the plasma membrane (9). A similar pattern of staining was reported for CHO cells over-expressing the PMCA4a (27).

Additional support for correct targeting of PMCA1a comes from the assay of PMCA activity using isolated microsomes. It was reported previously (15) that over-expression of human PMCA4 led to an increased Ca\(^{2+}\) uptake in microsomes prepared from COS-1 cells, and this activity is stimulated by oxalate. Stimulation by oxalate was interpreted as an evidence that PMCA4 was retained in the ER (15). An assay of Ca\(^{2+}\) uptake in microsomes obtained from control and PMCA1a over-expressing RAECs indicated that over-expression of PMCA1a led to an increase in the Ca\(^{2+}\) uptake that is not stimulated by oxalate (see below). Thus the combined evidence from immunoperoxidase staining and the properties of microsomal Ca\(^{2+}\) uptake suggest that the expressed PMCA1a is not retained in the ER but rather localized to the plasma membrane.

To determine whether the expressed protein was functional, the properties of ATP-dependent Ca\(^{2+}\) uptake were measured. A 70-fold excess concentration of thapsigargin (Tg, 200 nM) was included in the uptake reaction to ensure complete inhibition of SERCA pump activity, without affecting the calmodulin-dependent Ca\(^{2+}\) uptake mediated by the PMCA (15). As shown in Fig. 3A, the rate of ATP-dependent Ca\(^{2+}\) uptake in the membrane preparation from the PMCA-transfected cells (clone 8) was about 3-fold higher than the vector transfected controls (0.47 versus 0.16 nmol/mg/min). Similar results were obtained with microsomes prepared from clone 35 (not shown). Thus the activity assay shows that the over-expressed PMCA1a is functional in Ca\(^{2+}\) transport.

A critical parameter for the correct functioning of the PMCA is its affinity for Ca\(^{2+}\) and response to calmodulin. Therefore, Ca\(^{2+}\) transport activities of microsomes isolated from the transfected cells were tested as a function of free Ca\(^{2+}\) concentration and in the presence or absence of calmodulin. As shown in Fig. 3B, in membranes prepared from the over-expressing cells (clone 8), the activity of PMCA1a was dependent on Ca\(^{2+}\) concentration. Addition of calmodulin greatly enhanced the activity. Thus, the expressed isoform 1a shows a characteristic dependence on Ca\(^{2+}\) and calmodulin for activation. Comparison of the PMCA over-expressing cells (Fig. 3B) with the vector-transfected cells (Fig. 3C) indicated a 3-4-fold increase in PMCA activity at low and saturating Ca\(^{2+}\) concentrations.

Over-expression of PMCA Modifies the \([Ca^{2+}]_i\) Signal of RAEC Cells—To evaluate the role of PMCA1a in Ca\(^{2+}\) homeostasis and in \([Ca^{2+}]_i\), signaling, we measured \([Ca^{2+}]_i\) in resting cells and cells treated with the purinergic agonist ATP or the SERCA pump inhibitor thapsigargin (Tg). Fig. 4 depicts examples of the \([Ca^{2+}]_i\), signals of single control and clone 8 cells stimulated with ATP or Tg. In both cell types ATP caused a rapid increase in \([Ca^{2+}]_i\), which then stabilized at an elevated level, indicative of Ca\(^{2+}\) release from internal stores and sub-
cells were exposed to a mixture of ATP and Tg to discharge most of the agonist-mobilizable Ca\(^{2+}\) pool, [Ca\(^{2+}\)], increased to about 358 ± 21 (n = 46) nM, ATP and Tg increased [Ca\(^{2+}\)] of clone 8 cells only to 266 ± 22 (n = 37) nM. The difference in peak [Ca\(^{2+}\)], was highly significant (p < 0.01, Student’s t-test).

To exclude the possibility that modification of signal transduction was responsible for the reduced response to agonist we estimated the size of the intracellular Ca\(^{2+}\) pool with the Ca\(^{2+}\)-ionophore ionomycin (Fig. 5, b and d). Supermaximal concentration of ionomycin (10 \(\mu\)M) increased [Ca\(^{2+}\)] of Neo controls by 668 ± 43 (n = 4) nM and that of clone 8 cells by 372 ± 19 (n = 4) nM.

An additional finding illustrated in Fig. 5, a and c, is that the second phase increase in [Ca\(^{2+}\)], appears to be somewhat more prominent in clone 8 cells, suggesting a possible difference in Ca\(^{2+}\) influx rate. It is difficult to estimate the difference in plasma membrane Ca\(^{2+}\) pumping from the rate of reduction in [Ca\(^{2+}\)], after ATP and Tg treatment, since the agonists increased [Ca\(^{2+}\)], to different levels in the two cell types and Ca\(^{2+}\) influx appears to be modified by over-expression of the pump. Nonetheless, removal of external Ca\(^{2+}\) with EGTA caused faster reduction in [Ca\(^{2+}\)] in clone 8 relative to control. Although not conclusive, this provides an indication that the rate of Ca\(^{2+}\) pumping across the plasma membrane of clone 8 cells was higher than in controls.

Due to the apparent multiple effects of PMCA1a over-expression on Ca\(^{2+}\) signaling we proceeded to examine the expression and activity of the different Ca\(^{2+}\) transporting pathways involved in regulating [Ca\(^{2+}\)] (18, 19).

Over-expression of PMCA1a Down-regulates SERCA—In a previous successful over-expression of PMCA4b in CHO cells it was shown that PMCA4b expression reduced the formation of a phosphorylated intermediate by the SERCA pumps (27), suggesting reduced SERCA pump activity due to PMCA4b expression. We confirmed this finding by showing similar reduction in phosphoenzyme formation by the SERCA pumps of clone 8 and 35 cells (not shown). We proceeded to extend this finding by testing Ca\(^{2+}\) pumping activity, transcription and translation of SERCA pumps in control and PMCA1a over-expressing cells. Fig. 6A shows that SERCA-mediated Ca\(^{2+}\) uptake was reduced by about 35% in microsomes isolated from PMCA1a over-expressing cells. Further evidence is given in Fig. 8, A and B, below showing reduced Ca\(^{2+}\) pumping activity in permeabilized clone 8 cells. Northern blot analysis of total RNA using a probe representing SERCA 3 showed more than 50% reduction in SERCA 3 mRNA levels in clone 8 cells (Fig. 6B). Finally, Western blot analysis using an antibody that recognizes SERCA pumps showed a reduced level of SERCA protein in clone 8 cells (Fig. 7, upper panel). Hence, over-expression of PMCA1a cDNA resulted in lower amount of SERCA mRNA and protein to reduce Ca\(^{2+}\) uptake into the ER.

Over-expression of PMCA1a Down-regulates IP\(_3\)-R—The next pathway examined was Ca\(^{2+}\) release from internal stores. As shown in Fig. 7 (lower panel) over-expression of PMCA1a pump down-regulated the abundance of the IP\(_3\)-R protein. The reduction in IP\(_3\)-R protein level was comparable to that found for the reduction in SERCA pump protein (Fig. 7).

To evaluate the impact of reduced levels of the IP\(_3\)-activated channel on Ca\(^{2+}\) release we used streptolysin O (SLO)-permeabilized cells and measured Ca\(^{2+}\) uptake and release in the presence of mitochondrial inhibitors. Under these conditions Ca\(^{2+}\) uptake was completely dependent on the presence of ATP in the permeabilization medium and was inhibited better than 96% by 0.1 \(\mu\)M Tg (not shown). Fig. 8, A and B, shows that the rate of Ca\(^{2+}\) uptake by clone 8 cells was slower by about 38 ± 1.2% (n = 9) compared to that measured in control cells. In
addition, clone 8 cells reduced medium \([\text{Ca}^{2+}]\) to 307 ± 26 nM \((n = 9)\) as compared to control cells, which reduced medium \([\text{Ca}^{2+}]\) to 203 ± 15 nM \((n = 9)\). These results further support the reduced SERCA pump activity in clone 8 cells.

After stabilization of medium \([\text{Ca}^{2+}]\) the permeabilized cells were used to measure the properties of IP_{3}-mediated \(\text{Ca}^{2+}\) release. Due to differences in SERCA pump activity between the cells, the ability of IP_{3} to release \(\text{Ca}^{2+}\) was expressed as total \(\text{Ca}^{2+}\) release (Fig. 8C) and as a percentage of \(\text{Ca}^{2+}\) mobilized by ionomycin (Fig. 8D). It can be seen that in both cell types IP_{3} released about 50% of the ionomycin-mobilizable \(\text{Ca}^{2+}\) pool with similar apparent affinity. This would suggest that the IP_{3}R remaining in clone 8 cells are not modified and that they are at sufficiently high density to allow maximal \(\text{Ca}^{2+}\) release.

The lower density of IP_{3}R in clone 8 cells prompted testing the rate of IP_{3}-mediated \(\text{Ca}^{2+}\) release. Fig. 9 shows that \(\text{Ca}^{2+}\) release in clone 8 cells was slower than that of control cells. In four determinations from two experiments the rate in clone 8 was reduced by an average of 34 ± 4%.

Over-expression of PMCA1a Up-regulates CRAC Activity—To study the influence of PMCA1a expression on \(\text{Ca}^{2+}\) release-activated \(\text{Ca}^{2+}\) influx (CRAC), cells in \(\text{Ca}^{2+}\)-free medium were stimulated with a mixture of Tg and ATP. After return of \([\text{Ca}^{2+}]_i\) to resting levels \(\text{Ca}^{2+}\) influx was initiated by addition of 1.5 mM \(\text{Ca}^{2+}\) to the medium. Fig. 10 shows that the initial rate of \(\text{Ca}^{2+}\) influx into clone 8 cells was faster than that into Neo control. In control cells the rate averaged 4.55 ± 0.36 \((n = 4)\) nM/s, whereas in clone 8 cells the rate averaged 10.52 ± 0.96 \((n = 4)\) nM/s. Since changes in \([\text{Ca}^{2+}]_i\) represent the sum of the activity of all \(\text{Ca}^{2+}\) transport pathways, the rate of divalent cation influx was further evaluated by measuring the rate of unidirectional Mn^{2+} influx under the same conditions (Fig. 10, b and d). Mn^{2+} influx in clone 8 cells was about 2.63 ± 0.13 \((n = 4)\) fold faster than measured in Neo controls.

Measurement of External [Ca^{2+}]

The multiple effect of PMCA1a expression on the \(\text{Ca}^{2+}\) transporting pathways, the different levels of \([\text{Ca}^{2+}]_i\) increase caused by agonists in the two cell types and the fact that \([\text{Ca}^{2+}]_i\) measurement reflects the sum of the activity of all pathways made it impossible to evaluate the rate of \(\text{Ca}^{2+}\) pumping across the plasma membrane by following \([\text{Ca}^{2+}]_i\). To overcome these difficulties we measured...
shown). Similar results were obtained in one additional experiment. The blots were hybridized with a probe to show the equal loading of RNA samples. Similar reductions in SERCA mRNA (4.4 kilobases) was identified by using a specific cDNA probe representing SERCA 3, the major species in RAEC (T. H. Kuo, unpublished data). The same blot was hybridized with an actin cDNA probe to show the equal loading of RNA samples. Similar reductions in SERCA mRNA were obtained in four additional experiments using clone 8 or 35 cells.

**DISCUSSION**

Although the biochemical properties of the PMCA have been extensively studied (1), its functional role in resting and stimulated cells is not well understood. In recent years it became clear that the pump plays a pivotal role in the down-stroke phase of the [Ca\(^{2+}\)] signal evoked by agonist stimulation (2, 18, 19) and during [Ca\(^{2+}\)] oscillations (7). Direct (2, 5) and indirect (4, 6) evidence suggests that the PMCA is activated by agonists, and the activation is accompanied by phosphorylation of the pump protein (8, 9).

In an effort to better understand the role and regulation of the PMCA in Ca\(^{2+}\) signaling, we over-expressed the PMCA1a isoform and isolated stable transfecant. A major difficulty in using expression systems to study the role of PMCA in Ca\(^{2+}\) transport in intact cells was the correct targeting of the pump to the plasma membrane (14). The cell line, vector, transfection, and selection procedures used in the present studies overcame this problem. Two types of evidence suggest proper targeting of most of the PMCA1a to the plasma membrane. Immunocytochemistry showed a typical punctate staining in the plasma membrane of over-expressing cell lines with minimal cytosolic staining. The high levels of PMCA1a colocalized with markers of caveolae, similar to the localization of native pump protein reported before (28–30). The increased Ca\(^{2+}\) pumping activity in microsomes of PMCA1a over-expressing cells was independent of Tg and oxalate treatment, and the activity was accompanied by phosphorylation of the pump protein (8, 9).

The successful over-expression of high levels of PMCA1a in the plasma membrane revealed that pump activity can be regulated on several levels. The first finding of note was that agonist stimulation activates the plasma membrane Ca\(^{2+}\) pump (2). Thus, it was important to show that the faster Ca\(^{2+}\) pumping measured in clone 8 cells is independent of cell stimulation. Fig. 11, c and d, shows that when internal Ca\(^{2+}\) was mobilized by ionomycin, the initial rate of Ca\(^{2+}\) extrusion by clone 8 cells was about 1.35 ± 0.03 (n = 3) fold higher than that of Neo controls. This was despite the lower increase in [Ca\(^{2+}\)] caused by ionomycin in clone 8 cells (Fig. 5). This experiment also confirmed the reduced size of the internal stores Ca\(^{2+}\) pool in clone 8 cells.

**FIG. 6.** **SERCA pump activity and mRNA levels.** Panel A, ATP-mediated, oxalate-dependent Ca\(^{2+}\) uptake was measured by incubating microsomes in \(^{45}\)Ca uptake medium with and without Tg and in the presence of 7.5 mM oxalate. At the indicated times samples were removed to analyze \(^{45}\)Ca content in microsomes. The figure shows the fraction of the Tg-sensitive \(^{45}\)Ca uptake. Panel B shows a Northern blot analysis of total RNA prepared form Neo control and clone 8 cells. SERCA mRNA (4.4 kilobases) was identified by using a specific cDNA probe representing SERCA 3, the major species in RAEC (T. H. Kuo, unpublished data). The same blot was hybridized with an actin cDNA probe to show the equal loading of RNA samples. Similar reductions in SERCA mRNA were obtained in four additional experiments using clone 8 or 35 cells.

**FIG. 7.** **Western blot analysis of SERCA and IP\(_{R}\) proteins.** Lysates prepared from Neo control and clone 8 cells were separated by SDS-polyacrylamide gel electrophoresis and Western blotted. SERCA protein (100 kDa) and IP\(_{R}\) (250 kDa) were identified on separate blots by using specific antibody followed by a chemiluminescence methods. SERCA antibody (monoclonal anti-SERCA2, clone 2A7-A1 which cross-reacts with SERCA3) was purchased from Affinity BioReagents and IP\(_{R}\) (type 1) antibody was a gift from Dr. Richard J. H. Wągckiewicz, State University of New York at Syracuse. The blots were hybridized with an actin cDNA probe to show the equal loading of RNA samples. Similar results were obtained in one additional experiment.

The successful over-expression of high levels of PMCA1a in the plasma membrane revealed that pump activity can be regulated on several levels. The first finding of note was that despite the correct targeting and over-expression of the PMCA1a, resting [Ca\(^{2+}\)] was identical in control cells and cells of the different clones over-expressing the PMCA1a. Since resting [Ca\(^{2+}\)] is determined exclusively by pump/leak turnover across the plasma membrane (18), it was clear that either the activity of the over-expressed pumps was down-regulated in intact cells, Ca\(^{2+}\) influx rate was much higher in cells over-expressing the pump or a combination of both. Measurement of Ca\(^{2+}\) and Mn\(^{2+}\) influx into resting cells (such as those in Fig. 10, a and b) did not reveal major differences in Ca\(^{2+}\) influx rate among the different cell types. Therefore, it was likely that the over-expressed pumps were not active at resting [Ca\(^{2+}\)].

Following [Ca\(^{2+}\)]\(_{o}\) to estimate the rate of uni
directional Ca\textsuperscript{2+} efflux revealed that the activity of the over-expressed PMCA pumps was also regulated during agonist stimulation and/or when [Ca\textsuperscript{2+}]\textsubscript{i} was high. Thus, Western blot analysis showed about 4-fold increase in pump protein in the over-expressing clones used for the present studies (clone 8 and 35). The over-expressed pumps were fully functional as they increased Ca\textsuperscript{2+} uptake in isolated microsomes, had the expected apparent affinity for Ca\textsuperscript{2+}, and were regulated by calmodulin. Yet, the rate of Ca\textsuperscript{2+} pumping in intact clone 8 and clone 35 cells was about 1.4-fold over that measured in control cells. Our measurements of Ca\textsuperscript{2+} pumping activity are not influenced by the activity of other Ca\textsuperscript{2+} transporting pathways since we measured net Ca\textsuperscript{2+} efflux by following [Ca\textsuperscript{2+}]\textsubscript{out}. The lower than expected increase in Ca\textsuperscript{2+} pumping in intact cells is also not because the lower increase in [Ca\textsuperscript{2+}]\textsubscript{i} evoked by agonist in these cells. When dCa\textsuperscript{2+}/dt for the two cell types is calculated at the same [Ca\textsuperscript{2+}]\textsubscript{i} from experiments similar to those in Figs. 10, b and d, and 11, the increased rate of pumping due to PMCA1a over-expression is at best 1.67-fold over control. Our findings are further supported by the study of Guerini et al. (27). These authors reported a 20-fold increase in Ca\textsuperscript{2+} pumping activity in microsomes prepared from CHO cells over-expressing PMCA4b. Recalculation of the data in their Fig. 5 shows that such over-expression of active pump protein in the plasma membrane caused only about a 1.7-fold increase in \textsuperscript{45}Ca\textsuperscript{2+} efflux rate (27). Considering the fact that agonist-induced \textsuperscript{45}Ca efflux is influenced by reuptake of Ca\textsuperscript{2+} into the ER and reflects the sum of Ca\textsuperscript{2+} extrusion and Ca\textsuperscript{2+}/Ca\textsuperscript{2+} exchange through the CRAC channel (18), the 1.7-fold increased rate of \textsuperscript{45}Ca efflux is an overestimation of the increase in Ca\textsuperscript{2+} pump rate (27).

The finding discussed above of the two independent studies, using different cell type and different pump isoforms, suggests that cells are capable of controlling Ca\textsuperscript{2+} pumping rate independent of the number of the Ca\textsuperscript{2+} pumps in the plasma membrane. Hence, a 4- (present studies) or a 20- (Ref. 27) fold increase in the number of Ca\textsuperscript{2+} pumps increased Ca\textsuperscript{2+} pumping rate in intact cells to similar extent (1.4–1.7-fold). This suggests a set point for the maximal possible Ca\textsuperscript{2+} pumping rate which is compatible with cell survival, since over-expression of PMCA strongly affects the timing of the cell cycle (27). How the cells achieve such a set point is not clear at present. Two obvious possibilities are down-regulation of pumping activity and/or shielding the pumps from the increase in [Ca\textsuperscript{2+}] by compartmentalizing the Ca\textsuperscript{2+} pool and the areas of Ca\textsuperscript{2+} release (31–37). Discriminating between these and other possibilities requires further studies. Using the PMCA over-expressing cells it should be now possible to address such questions.

Another major and significant finding of the present study...
wastheadaptiveregulationofallthemajorCa\textsuperscript{2+} transportinpaths in response to over-expression of the PMCA1a. The activity of the CRAC pathway in the plasma membrane was up-regulated by as much as 2.6-fold, significantly more than the net increase in Ca\textsuperscript{2+} pumping activity. The large increase in CRAC activity is likely to compensate for the increased Ca\textsuperscript{2+} content (see below). It is interesting that activation of CRAC in the PMCA1a over-expressing cells still required Ca\textsuperscript{2+} release from internal stores, indicating maintained regulation of the CRAC pathway by Ca\textsuperscript{2+} content of the ER (18, 38). Since nothing is known about the nature of the CRAC pathway (38) we could not determine on what level CRAC activity was regulated in PMCA1a over-expressing cells.

Over-expression of PMCA1a down-regulated the contribution of the internal Ca\textsuperscript{2+} pool to the Ca\textsuperscript{2+} signal by reducing the level and activity of both the SERCA pumps and the IP\textsubscript{3}R. Previous studies similarly showed that over-expression of PMCA4b in the plasma membrane reduced the phosphorylated intermediate formed during the turnover cycle of the SERCA pump in CHO cells (27). Here we show that the reduced activity was largely due to a reduction in the number of the pumps. Also in the case of IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release, the reduced rate of release could be attributed to reduced number of IP\textsubscript{3}R, with normal apparent affinity for IP\textsubscript{3}. The reduction in the number of SERCA pumps resulted in reduced rate of Ca\textsuperscript{2+} pumping and Ca\textsuperscript{2+} content in the ER. This was the case whether Ca\textsuperscript{2+} content in the ER was measured in permeable or intact cells and whether it was released by IP\textsubscript{3} or ionomycin. The reduced Ca\textsuperscript{2+} content in the ER was reflected in a reduced initial agonist-evoked [Ca\textsuperscript{2+}]\textsubscript{i} increase.

The regulation of the different Ca\textsuperscript{2+} transporting pathways by over-expression of PMCA1a appears to be on the gene level. In the case of the SERCA pump we were able to show that over-expression of PMCA1a reduced SERCA gene expression by more than 50%, which is likely to account for the reduced amount of SERCA pump protein in these cells. The mechanism by which PMCA1a expression regulates the SERCA gene(s) is not clear at present. Up-regulation of SERCA2 gene by the hormone T3 and by the platelet-derived growth factor have been reported in cardiac (39) and smooth muscle cells (40), respectively. Similarly, we have demonstrated the stimulation of SERCA3 gene expression (the major isoform) in RAEC by epidermal growth factor and angiotensin II (41). SERCA3 gene is also up-regulated in platelets from hypertensive rats (42). In fibroblasts, the expression of a distinct Tg-resistant Ca\textsuperscript{2+} pump is induced by continuing exposure to Tg (43, 44). These findings together with the present studies, suggest that cells can regulate the level of SERCA in response to external stimuli or a change in the level or activity of the other Ca\textsuperscript{2+} transport pathways.

In summary, the present studies demonstrate that functional over-expression of PMCA1a led to a down-regulation of SERCA gene expression, a reduction in the number of IP\textsubscript{3}R, and an increase in the activity of the CRAC pathway. It is not clear at present whether the regulation of all pathways was on the gene level. However, if this is the case, it is possible that the genes of all the major Ca\textsuperscript{2+} transporting pathways are regula-
torally linked to concomitantly modulate the activities of all pathways. The link can be provided by the \([\text{Ca}^{2+}]\) levels in resting cells. Considering the information encoded in the \([\text{Ca}^{2+}]\) signal and the involvement of \(\text{Ca}^{2+}\) in numerous vital cell functions, it is not surprising that its concentration is regulated on several levels. Regulation on the level of gene expression is likely to provide a constant \([\text{Ca}^{2+}]\) signal, which is essential for cell survival. This is emphasized in several recent studies showing the modulation of the cell cycle (27, 43, 44) by over-expression of PMCA pumps and/or down-regulation of SERCA pumps by Tg treatment.

Acknowledgments—We thank Gary Shull for providing rat PMCA1a cDNA, P. Tabaeska and Ken Palmer for helping with the immunoperoxidase studies.

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