Regulation of SERCA Ca\(^{2+}\) pump expression by cytoplasmic [Ca\(^{2+}\)] in vascular smooth muscle cells

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Wu, Kwan-Dun, David Bungard, and Jonathan Lytton. Regulation of SERCA Ca\(^{2+}\) pump expression by cytoplasmic [Ca\(^{2+}\)] in vascular smooth muscle cells. Am J Physiol Cell Physiol 280: C843–C851, 2001.—Vascular smooth muscle cells (VSMC) express three isoforms of the sarcoplasmic or endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump; SERCA2b predominates (91%), whereas SERCA2a (6%) and SERCA3 (3%) are present in much smaller amounts. Treatment with thapsigargin (Tg) or A-23187 increased the level of mRNA encoding SERCA2b four- to fivefold; SERCA3 increased about 10-fold, whereas SERCA2a was unchanged. Ca\(^{2+}\) chelation prevented the Tg-induced SERCA2b increase, whereas Ca\(^{2+}\) elevation itself increased SERCA2b expression. These responses were discordant with those of 78-kDa glucose-regulated protein/immunoglobulin-binding protein (grp78/BiP), an endoplasmic reticulum stress-response protein. SERCA2b mRNA elevation was much larger than could be accounted for by the observed increase in message stability. The induction of SERCA2b by Tg did not require protein synthesis, nor was it affected by inhibitors of calcineurin, protein kinase C, Ca\(^{2+}\)/calmodulin-dependent protein kinase, or tyrosine protein kinases. Treatment with the nonselective protein kinase inhibitor H-7 prevented Tg-induced SERCA2b expression from occurring, whereas another nonselective inhibitor, staurosporine, was without effect. We conclude that changes in cytosolic Ca\(^{2+}\) control the expression of SERCA2b in VSMC via a mechanism involving a currently uncharacterized, H-7-sensitive but staurosporine-insensitive, protein kinase.

thapsigargin; ionophore A-23187; mRNA; Northern blotting; kinase

A CHANGE IN THE CYTOPLASMIC concentration of Ca\(^{2+}\) acts as an ubiquitous signal to control a host of diverse physiological events, ranging from fertilization and cell proliferation to muscle contraction and apoptosis (3). In smooth muscle cells, a rise in the global intracellular Ca\(^{2+}\) concentration generates contraction and muscle tone via the calmodulin-dependent activation of myosin light chain kinase (41). Local changes in Ca\(^{2+}\) close to the plasma membrane, however, have been shown to induce hyperpolarization and, consequently, smooth muscle relaxation (15). In addition, Ca\(^{2+}\) signals are essential events in agonist-induced hypertrophic growth and in proliferation of vascular smooth muscle (3, 29). Precise temporal and spatial control over cytoplasmic Ca\(^{2+}\) is thus essential for maintaining normal cell function and homeostasis.

The main site for dynamic control of cellular Ca\(^{2+}\) homeostasis is the sarcoplasmic or endoplasmic reticulum. Here, following the activation of selective signaling pathways, Ca\(^{2+}\) can be released into the cytoplasm through inositol trisphosphate- or ryanodine-receptor channels. The Ca\(^{2+}\) signal is then terminated largely by resequestration of Ca\(^{2+}\), mediated via an ATP-dependent Ca\(^{2+}\) pump. Molecular cloning studies have revealed a family of three genes that encode sarcoplasmic or endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pumps. SERCA1a and SERCA1b are alternatively spliced products expressed exclusively in fast-twitch skeletal muscle in a developmentally regulated manner (5). Cardiac and slow-twitch muscle express the SERCA2a gene product whereas an alternatively spliced isoform, SERCA2b, is expressed ubiquitously (25). SERCA3 is expressed in a selected number of tissues actively involved in secretion (6, 37, 43). Blood vessels express SERCA2b and, to a lesser extent, SERCA2a in the smooth muscle layer and SERCA3 and SERCA2b in the endothelial cells.

SERCA pump activity lowers the concentration of Ca\(^{2+}\) in the cytoplasm while at the same time raising that of the sarcoplasmic/endoplasmic reticulum. The content of Ca\(^{2+}\) within the sarcoplasmic/endoplasmic reticulum controls other processes, such as the sensitivity of Ca\(^{2+}\) release and the activity of plasma membrane entry channels. Consequently, SERCA activity has a very direct and dynamic effect on patterns of Ca\(^{2+}\) signaling and the cellular events these signals...
control (3, 4). For example, treatment of cultured smooth muscle cells with the SERCA-specific inhibitor, thapsigargin (Tg), results in dramatic effects on cell growth (42). In the heart, alterations in SERCA activity induced by various transgenic models have profound effects on contractility (10, 24). Gene-targeted ablation studies have also demonstrated an essential role for SERCA2 in heart function (35) and for SERCA3 in endothelial- or epithelial-dependent relaxation of smooth muscle (16, 22).

Several human diseases are also associated with changes in SERCA expression. Brodie’s disease, which is manifested as a defect in skeletal muscle relaxation, is associated with mutations in SERCA1 (33). Darier’s disease, an autosomal-dominant skin disorder, has been shown to result from mutations in the SERCA2 gene (40). Additionally, changes in SERCA expression have been associated with heart failure in humans and with animal models of heart disease, hypertension, diabetes, and aging (23, 31). Moreover, changes in the expression of SERCA genes in smooth muscle have been reported in response to platelet-derived growth factor (28).

Despite the broad-ranging studies connecting SERCA function and cellular Ca\(^{2+}\) homeostasis and the clear link between changes in SERCA function and the etiology of cardiovascular disease, examination of the factors that regulate SERCA expression has been rather sporadic and limited mainly to thyroid hormone (12, 46). In this report, we investigated regulation of SERCA expression in vascular smooth muscle cells (VSMC) and identified cytoplasmic Ca\(^{2+}\) as a key controlling factor.

**METHODS**

Cell culture and isolation of total RNA. VSMC were isolated from the thoracic aorta of 200- to 250-g male Sprague-Dawley rats by enzymatic dispersion (14). The cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamate, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin and were passaged once a week by harvesting about 1 \(\times\) 10\(^4\) cells/cm\(^2\). They were characterized as smooth muscle cells by positive immunofluorescent staining with a -actin antibody (Sigma, St. Louis, MO). Cells were plated onto 10-cm culture dishes each experiment, cells were plated onto 10-cm culture dishes and analyzed for all experiments. For each experiment, cells were plated onto 10-cm culture dishes and allowed to reach confluence. At that point, the medium was changed to one containing 1% fetal bovine serum for another 24 h before the experiments were started.

RNA was purified from each 10-cm dish by the acid guanidinium thiocyanate-phenol-chloroform extraction method. The yield from each plate was between 75 and 150 \(\mu\)g total RNA. Ten micrograms of each sample were fractionated on a 1% agarose gel containing 0.63 M formaldehyde as denaturant, transferred to a nylon membrane by capillary diffusion overnight, and fixed in place by ultraviolet irradiation (1). Equal loading of each lane on these gels was confirmed by the intensity of ethidium bromide staining of the ribosomal RNA bands and, in some cases, by hybridization to a rat glyceraldehyde-3-phosphate dehydrogenase probe.

**Northern blot analysis.** The blots were hybridized and washed at high stringency with digoxigenin-UTP-labeled antisense riboprobes according to instructions of the manufacturer (Roche Molecular Biochemicals), as previously described (44). Probes specific for each SERCA isoform were obtained from the 3′-untranslated region of the corresponding rat cDNA: SERCA2a, DraI (3591) to ClaI (3863); SERCA2b, XbaI (3758) to HaeIII (4197); and SERCA3, HindIII (3024) to PstI (3407). As described previously, these probes show cross-reaction (44). The glyceraldehyde-3-phosphate dehydrogenase antisense riboprobe spanned the full rat clone (GenBank accession no. X02231). The probe for 78-kDa glucose-regulated protein (grp78) (immunoglobulin-binding protein [BiP]) was obtained by random-primed \(^{32}\)P-labeling of the mouse cDNA (GenBank accession no. M30779). Hybridization with this probe was at 42°C but otherwise in a manner identical to that used for the digoxigenin probes. Chemiluminescent or radioactive signals were then visualized by exposure to X-ray film and quantified by computerized densitometry using NIH Image software (http://rsb.info.nih.gov/nih-image/) to obtain the relative RNA levels. In the case of SERCA, quantitative data comparing isoforms was obtained by analyzing synthetic transcripts from plasmid clones of known amounts in parallel with the VSMC RNA, as described previously (44).

**Protein labeling, immunoprecipitation, and immunoblotting.** Confluent monolayers of VSMC in 10-cm plates were treated as indicated, and \(^{35}\)S-methionine was added to the culture medium at 80 \(μ\)Ci/ml for 4 additional hours. The cells were then washed in phosphate-buffered saline and solubilized in 1 ml/10-cm dish RIPA buffer (1% Nonidet P-40 (NP-40), 0.5% deoxycholate, 0.15 M NaCl, 1 mM EDTA, and 25 mM Tris-HCl, pH 7.5) for 20 min on ice. This extract was then clarified by centrifugation for 10 min at maximum speed in an Eppendorf microcentrifuge, assayed for protein content using a detergent-compatible assay kit (Bio-Rad Laboratories, Hercules, CA), and stored frozen at −80°C until further analysis. For immunoblotts, aliquots of cell extracts prepared as above from unlabeled cells were separated on an SDS polyacrylamide gel, transferred to nitrocellulose, and probed using rabbit anti-SERCA C4 antibody (25). For immunoprecipitation, 0.1-ml aliquots of labeled cell extract were supplemented with SDS to 0.1%, then diluted with 0.9 ml of RIPA buffer containing 1 mg/ml bovine serum albumin. This solution was then clarified by centrifugation for 15 s in a microcentrifuge; the supernatant was removed to a fresh tube and incubated for 2 h at room temperature with 20 \(\mu\)g of either preimmune or immune rabbit anti-SERCA C4 antibody. Fifty microliters of IgG-Sepharose suspension was added, and incubation continued for 30 min on a rotator. This mixture was then centrifuged for 15 s, and the pellet was washed sequentially once each in RIPA buffer supplemented with 0.5 M NaCl and 0.1% SDS. Finally, the pellet was washed once with 0.1% NP-40, 1 mM EDTA, 25 mM Tris-HCl (pH 7.5); transferred to a fresh tube; recenterfuged; dissolved in 50 \(\mu\)l of sample buffer (20% glycerol, 6% SDS, 5% 2-mercaptoethanol, and 0.13 M Tris-HCl, pH 6.8); heated at 60°C for 2 min; and then resolved on a 7.5% Laemmli SDS polyacrylamide gel (19). Samples of untreated, labeled cell lysate were also run on a parallel gel. The dried gels were exposed to Kodak MR X-ray film to visualize the labeled protein bands.

**Reagents.** Pharmacological reagents were purchased from LC Laboratories (Woburn, MA), Seikagaku America (Falmouth, MA), or Calbiochem (San Diego, CA). Cell culture reagents were from Life Technologies (Rockville, MD), and other chemicals were of analytic or molecular biology grade or better.
Statistical analysis. Comparison of groups in experiments with several different treatments was performed using ANOVA followed by Dunnett’s t-test. For the quantitative immunoblot data of Fig. 3B, a replicated two-factor ANOVA analysis was performed.

RESULTS

Rat aortic VSMC cultured under normal conditions express a mixture of SERCA isoforms 2a, 2b, and 3, although SERCA2b accounts for >90% of the total SERCA transcripts (Fig. 1). Cellular Ca^{2+} homeostasis can be dramatically influenced using the SERCA-specific inhibitor Tg or the Ca^{2+} ionophore A-23187, both of which deplete intracellular Ca^{2+} stores of the sarcoplasmic/endoplasmic reticulum and elevate cytosolic Ca^{2+} (27, 45). Treatment of VSMC with either Tg or A-23187 induced a remarkable upregulation of SERCA mRNA (Figs. 1 and 2). As reported previously by others (11), we noted that under our conditions neither Tg nor ionophore treatment was lethal but did induce a profound, although transient, reduction in proliferation of nonconfluent cells (data not shown). Examination of the response of individual SERCA isoform transcripts to Tg treatment revealed about a fourfold increase in SERCA2b, no change in SERCA2a, and an ~10-fold increase in SERCA3 (Fig. 1). Note that the common 8-kb transcript observed with both SERCA2a and SERCA2b probes encodes a SERCA2b protein, whereas unique 4.5-kb transcripts encode SERCA2a and SERCA2b proteins (6). The pattern of SERCA isoform induction produced by ionophore treatment was virtually identical to that observed with Tg (data not shown). Consequently, further experiments were focussed on regulation of SERCA2b expression.

![Fig. 1](https://example.com/fig1.png)

Fig. 1. Sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase (SERCA) isoform expression in rat vascular smooth muscle cells (VSMC). Cultured cells were treated for 10 h with either 0.5 μM thapsigargin (Tg) or 0.1% DMSO vehicle control alone (C). Total RNA was isolated from these cells, and 10-μg samples were separated on an agarose-formaldehyde gel, transferred to a nylon membrane, and then hybridized with probes for SERCA2a (S2a), SERCA2b (S2b), SERCA3 (S3) or glyceraldehyde-3-phosphate dehydrogenase (GPDH). A: a representative experiment analyzed by Northern blot is presented. Size markers in kilobases are indicated at left. B: quantitative data averaged from 3 independent experiments such as the one shown in A indicating the relative amounts (Rel) of mRNA encoding each SERCA isoform, normalized to SERCA2b expression under control conditions. The data are also recalculated to show the percent contribution of each isoform to total SERCA transcript content under each condition (%).

![Fig. 2](https://example.com/fig2.png)

Fig. 2. Induction of SERCA2b by Tg or Ca^{2+}-ionophore. Cultured rat VSMC were treated either for varying time (A) with 0.5 μM Tg (○, n = 10), 1 μM ionophore A-23187 (■, n = 4), or 0.1% DMSO control (□, n = 3); or for 6 h with varying doses (B) of Tg (○, n = 4) or A-23187 (■, n = 4). RNA was isolated from individual 10-cm plates and analyzed for SERCA2b mRNA expression by quantitative Northern blotting. Average values ± SE are plotted as SERCA2b content relative to time zero (A) or as the fold increase expressed as a percent of the maximum response (B).
pendent experiments. Representative of 2 independent experiments.

Arrowheads indicate bands whose expression is induced by Tg treatment was used in most experiments. The Tg-treated group was statistically different from the control group (P < 0.01), and there was no interaction with time. C: 20-µg samples of extracts from cells were subject to immunoblot analysis with the anti-SERCA antibody, C4. Arrowhead indicates SERCA band. A representative blot from 3 different experiments is shown. B: averaged data ± SE from 3 experiments such as the one shown in A are plotted as the Tg-induced level of SERCA relative to control for the indicated times. The Tg-treated group was statistically different from the control group (P < 0.01), and there was no interaction with time. C: 20-µg samples of extracts from [35S]-labeled cells treated for either 6 or 16 h with or without Tg were separated on an SDS polyacrylamide gel. Arrowheads indicate bands whose expression is induced by Tg treatment. D: 0.1-ml samples of extracts from [35S]-labeled cells treated for the indicated times with Tg were subjected to immunoprecipitation with either C4 (I) or preimmune serum (PI). Arrowhead shows position of band corresponding to SERCA. Representative of 2 independent experiments.

particularly with respect to the concentration of serum present (data not shown). Presumably this is due to the very hydrophobic nature of these compounds and the binding properties of serum proteins. Care was taken, therefore, to ensure that all experiments were performed under identical conditions and used 1% bovine serum albumin from the same lot. Induction of SERCA2b message was a relatively slow process, generally peaking with a four- to fivefold increase in abundance at ~6–10 h. Induction remained stable past 16 h and then declined at 24 h and later. The decline in SERCA2b abundance at later time points was found to be mirrored by other messages, and thus 6–16 h of treatment was used in most experiments.

The influence of Tg treatment on SERCA protein expression was analyzed using a polyclonal anti-SERCA antibody, C4 (25), as illustrated in Fig. 3. Immunoblotting of extracts from treated VSMC showed a modest, but statistically significant, increase in SERCA abundance (Fig. 3, A and B). Labeling of newly synthesized proteins using [35S]methionine (Fig. 3C) revealed a dramatic reduction in overall protein translation, as anticipated for the unfolded-protein response induced by depletion of Ca²⁺ from the sarcoplasmic/endoplasmic reticulum (32). Prominent bands at 78 and 94 kDa presumably represent the endoplasmic reticulum chaperones, grp78 and grp94 (20). Immunoprecipitation of SERCA from these labeled extracts revealed no appreciable reduction and a possible modest increase in protein expression, consistent with the immunoblotting analysis. Thus, although SERCA2b transcripts were increased four- to fivefold by Tg treatment, the increase in SERCA protein expression was not as large. Nevertheless, these modest effects of Tg on SERCA protein expression were in contrast to the large reduction observed in overall protein synthesis. Similar results were obtained for A-23187 treatment (data not shown).

Because SERCA2b induction paralleled the induction of the endoplasmic reticulum stress-response proteins grp78 and grp94, we next investigated whether other means of depleting Ca²⁺ stores would also increase SERCA2b message. Chelating extracellular Ca²⁺ with EGTA or intracellular Ca²⁺ with the membrane-permeant Ca²⁺ chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM), has been shown to decrease the level of Ca²⁺ in both the cytosol and the endoplasmic reticulum stores (30). As shown in Fig. 4, treatment of

Fig. 3. Tg causes SERCA protein induction. Cultured rat VSMC were treated for varying time with either 0.5 µM Tg or 0.1% DMSO vehicle control (C). A: 20-µg samples of extracts from cells were subject to immunoblot analysis with the anti-SERCA antibody, C4. Arrowhead indicates SERCA band. A representative blot from 3 different experiments is shown. B: averaged data ± SE from 3 experiments such as the one shown in A are plotted as the Tg-induced level of SERCA relative to control for the indicated times. The Tg-treated group was statistically different from the control group (P < 0.01), and there was no interaction with time. C: 20-µg samples of extracts from [35S]-labeled cells treated for either 6 or 16 h with or without Tg were separated on an SDS polyacrylamide gel. Arrowheads indicate bands whose expression is induced by Tg treatment. D: 0.1-ml samples of extracts from [35S]-labeled cells treated for the indicated times with Tg were subjected to immunoprecipitation with either C4 (I) or preimmune serum (PI). Arrowhead shows position of band corresponding to SERCA. Representative of 2 independent experiments.

Fig. 4. Ca²⁺ chelation prevents induction of SERCA2b mRNA by Tg. Cultured rat VSMC were pretreated with either vehicle (○, n = 5); a single dose of 20 µM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM, □, n = 4); 20 µM BAPTA-AM followed by additional doses at 8 and 16 h (△, n = 2); 20 µM BAPTA-AM followed by a single additional dose at 8 h only (●, n = 2); or with 2 mM EGTA (□, n = 4). Thirty minutes after the initial additions, 0.5 µM Tg was added. RNA was prepared from plates of cells at timed intervals and then analyzed for SERCA2b content by quantitative Northern blotting. The data are plotted as fold induction relative to time 0 ± SE.
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Fig. 5. SERCA is not induced by Ca2+ store depletion or the stress response. Cultured rat VSMC were treated with DMSO vehicle control (C), 0.5 μM Tg, 1 μM ionophore A-23187 (A), 2 mM EGTA (E), Tg and EDTA (TgE), and 20 μM BAPTA-AM 30 min before and 8 h after Tg addition (Tg+E) or with 0.25% 2-mercaptoethanol (β-ME). RNA was prepared from cells 6 h (A), 4 h (B), or at the indicated number of hours after treatment (C) and analyzed for either SERCA2b or 78-kDa glucose-regulated protein (grp78)immunoglobulin-binding protein (GRP-78) expression as indicated by Northern blotting. Approximate size in kilobases of the grp78 transcript and of the two SERCA2b transcripts are shown. These data are representative of at least 2 independent experiments.

VSMC with 2 mM EGTA (which produces a free extracellular Ca2+ concentration of ~0.5 μM) completely abolished the Tg-induced increase in SERCA2b abundance. Treatment with a single 20-μM dose of BAPTA-AM resulted in a significant delay in the induction of SERCA2b mRNA. This delay was increased further by a second addition of BAPTA-AM at 8 h, and the increase in SERCA2b mRNA was completely prevented by further additions of BAPTA-AM every 8 h. Thus it appeared that, rather than inducing elevation of SERCA2b mRNA, reduction in store and cytosolic Ca2+ actually decreased expression.

The relationship between SERCA2b and grp78 induction was explored further by examining mRNA levels for these molecules in the same experiment. Figure 5A illustrates that, as anticipated, both grp78 and SERCA2b mRNA levels increased on treatment with either Tg or A-23187. Chelation of extracellular Ca2+, however, had dramatically opposite effects on grp78 mRNA, which increased, and SERCA2b mRNA, which remained the same or decreased. These differences were particularly evident when EGTA and Tg treatments were combined (Fig. 5B). Similar discordance was observed when cells were treated with both BAPTA-AM and Tg (Fig. 5C). The endoplasmic reticulum unfolded-protein response can be initiated not only by Ca2+ chelation but also by treatment with other agents, including 2-mercaptoethanol (20). As seen in Fig. 5C, treatment with 2-mercaptoethanol had no significant effect on SERCA2b levels, although there was a robust elevation of grp78 mRNA. Thus it appears that the signals responsible for the induction of SERCA2b mRNA are different from those involved in the endoplasmic reticulum unfolded-protein response.

Because chelation of Ca2+ prevented SERCA2b message elevation, we next investigated whether the rise in cytosolic Ca2+ induced by Tg was responsible for increasing SERCA2b expression. For these experiments, Ca2+ was modulated without Tg treatment. VSMC were cultured in medium supplemented with EGTA (expected to reduce Ca2+), with increased extracellular Ca2+, with ouabain (which is expected to reverse the Na+/Ca2+ exchanger and cause Ca2+ entry due to inhibition of the Na+-K+-ATPase and the subsequent rise in cytosolic Na+), or with caffeine (expected to induce Ca2+ release from the sarcoplasmic or endoplasmic reticulum through the ryanodine receptor). As shown in Fig. 6, all the treatments designed to raise cytosolic Ca2+ produced a significant increase in SERCA2b abundance, with the combination of ouabain and high extracellular Ca2+ producing the largest change. EGTA, on the other hand, reduced SERCA2b mRNA levels. These data are therefore consistent with the view that cytosolic Ca2+ itself controls SERCA2b expression.

Fig. 6. SERCA expression in response to altered cellular Ca2+ homeostasis. Cultured rat VSMC were treated with medium supplemented with 2 mM EGTA (free [Ca2+] ~0.5 μM, n = 4); vehicle alone ([Ca2+] = 1.8 mM, n = 4); 5 mM CaCl2 ([Ca2+] = 6.8 mM, n = 5); 10 mM caffeine (n = 4); 0.5 mM ouabain (n = 2); or 0.5 mM ouabain and 5 mM CaCl2 (n = 2). RNA was then isolated and analyzed for SERCA2b expression by quantitative Northern blotting. The data were pooled for 10- and 16-h treatments and are plotted as the ratio relative to control ± SE. All treated groups were statistically different from the control (*P < 0.01; #P < 0.03).
Elevation in steady-state message abundance, as seen for SERCA2b on Tg treatment, can come about through either an increase in transcription or a decrease in message degradation, or both. We therefore examined the stability of SERCA2b transcripts following Tg treatment, using actinomycin D to inhibit new mRNA synthesis. Figure 7 illustrates that, after 10 h of Tg treatment, the half-life of SERCA2b mRNA had increased from 9.5 to 17.3 h. This roughly twofold decrease in the rate of SERCA2b mRNA degradation could alone have produced only a twofold increase in steady-state SERCA2b message level. Because Tg treatment induced a four- to fivefold increase in SERCA2b message, this result implies that both transcription and mRNA degradation must have been altered.

Changes in cytosolic Ca$^{2+}$ influence a number of effector molecules and signaling pathways, particularly phosphorylation cascades (9). The involvement of various pathways downstream from Tg-induced changes in Ca$^{2+}$ was therefore investigated by the use of pharmacological reagents. The data are summarized in Fig. 8. First of all we tested whether the Tg response required new protein synthesis. Cycloheximide had no significant effect on the ability of Tg to induce SERCA2b mRNA, and thus we concluded that new protein synthesis was not necessary for the Tg response. The nonspecific kinase inhibitor H-7 was able to completely inhibit the effect of Tg, implicating a kinase pathway in the Tg response. Next, we tested whether direct activation of protein kinase C with phorbol myristate acetate or of protein kinase A with forskolin produced a change in SERCA2b mRNA. Neither of these agents had any effect by themselves, nor did they inhibit the SERCA2b elevation induced by Tg. Moreover, inhibition of protein kinase C, either by its downregulation after overnight treatment with phorbol myristate acetate or by acute treatment with staurosporine, had no effect on the Tg-induced elevation of SERCA2b mRNA. Treatment with the Ca$^{2+}$/calmodulin-dependent protein kinase inhibitor, KN-62; tyrosine kinase inhibitors bis-tyrphostin and genistein; or the calcineurin inhibitor cypermethrin were all without effect. Thus it appears that a protein kinase, unique among the commonly characterized ones, is involved in the regulation of SERCA2b expression. So far, we have been unable to identify this kinase.

**DISCUSSION**

We have demonstrated that VSMC in culture express three different SERCA Ca$^{2+}$ pump isoforms. SERCA2b was the predominant isoform, accounting for >90% of total SERCA transcripts, whereas SERCA2a and SERCA3 were present only in small amounts. The abundance of transcripts for SERCA2b and SERCA3, but not for SERCA2a, was dramatically increased on treatment with the SERCA-specific inhibitor Tg or the Ca$^{2+}$-ionophore A-23187. These agents act selectively to release the content of the sarcoplasmic/endoplasmic reticulum Ca$^{2+}$ stores. Depletion of the stores results in the activation of a plasma membrane Ca$^{2+}$ entry pathway, causing elevation of cytoplasmic Ca$^{2+}$ (4, 45). Further experiments with the Ca$^{2+}$ chelators, BAPTA and EGTA, as well as with other agents that elevate cytosolic Ca$^{2+}$ (high extracellular Ca$^{2+}$, caffeine, and ouabain) firmly established that in VSMC, SERCA mRNA levels did not increase...
as a consequence of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) depletion but instead responded to changes in cytosolic Ca\(^{2+}\). Furthermore, SERCA expression is discordantly regulated from that of the endoplasmic reticulum chaperone grp78. Regulation of SERCA expression in VSMC is thus distinct from the so-called stress response (32).

Two other reports have also examined regulation of SERCA expression by alterations to Ca\(^{2+}\) homeostasis induced by Tg (7, 17). Kuo et al. (17) concluded that, in bovine aortic endothelial cells, depletion of the endoplasmic reticulum Ca\(^{2+}\) stores was required for the induction of SERCA2 via increased transcription. The level of induction in that study (maximal 1.5-fold increase in SERCA2 mRNA) was rather modest compared with our observations in VSMC (4- to 5-fold increase). Caspersen et al. (7) reached a different conclusion when studying SERCA transcripts in the PC-12 cell line. These authors found that Tg and A-23187 induced a three- to fourfold increase in SERCA2b mRNA but had no effect on SERCA3 message levels. They concluded from these and other experiments that SERCA2b responded to the unfolded-protein stress mechanism, independent of the Ca\(^{2+}\) content of endoplasmic reticulum stores. Unlike our data from VSMC, SERCA2b regulation in PC-12 cells was dependent on new protein synthesis and did not involve message stability. Surprisingly, Tg had no effect on overall protein synthesis in their system. Such dramatically disparate results are an indication that SERCA expression is controlled by very different mechanisms in different cell types. It is possible that this may be due partly to inherent differences in Ca\(^{2+}\) homeostasis between immortalized cell lines and primary cultures. In any event, it is clear from these data that SERCA2b cannot be thought of as a generic stress-inducible protein.

The SERCA3 isoform is expressed at significant levels in only a selected number of tissues and appears to play an important role in Ca\(^{2+}\)-mediated stimulus-secretion coupling (6, 16, 22, 37). Biochemically, SERCA3 is characterized as having a much lower apparent affinity for Ca\(^{2+}\) than the other isoforms have (26). It is intriguing, therefore, that increasing cytosolic Ca\(^{2+}\) has, proportionately, the largest effect on SERCA3 expression. This suggests the possibility that sustained elevation of cytosolic Ca\(^{2+}\) might be a factor that contributes to the selective expression of SERCA3 in environments where it is suited to function.

The SERCA2a and 2b isoforms arise as a consequence of an alternative splicing process that results in transcripts of similar length (4.5 kb) that have different 3’-end exons (and consequently proteins with different carboxy termini). Additionally, an 8-kb transcript is present that is an unspliced read-through product, encoding a SERCA2b protein but having the 3’-untranslated region of the SERCA2a isoform further downstream (6). Both 8- and 4.5-kb SERCA2b transcripts increase four- to fivefold on treatment with Tg, whereas the 4.5-kb SERCA2a transcript is unaffected. Our data suggest that increases in transcription, as well as changes in message stability, contribute to the observed changes in mRNA level. Since all of the alternatively spliced SERCA2 transcripts are synthesized starting from the same promoter, it is intriguing that they are regulated independently. This suggests that the level of SERCA2a transcripts in cultured VSMC may be controlled by a splicing factor that is present in limitingly small amounts or a splicing repressor present in supersaturating amounts (2) and, therefore, that the production of the SERCA2a spliced product does not respond to changes in overall gene expression. Furthermore, our data suggest that elements responsible for the observed stabilization of mRNA must be found uniquely in the SERCA2b-specific 3’-end region. Previous studies examining the relationship between platelet-derived, growth factor-induced proliferation and SERCA isoform expression in pig aortic VSMC demonstrated a selective upregulation of SERCA2a (28). Clearly, selective regulatory pathways exist in VSMC for the independent control of SERCA isoform expression.

A rise in intracellular Ca\(^{2+}\) can lead to activation of a number of distinct signaling pathways that influence gene expression. These include protein kinase C, Ca\(^{2+}\)/calmodulin-dependent protein kinases, tyrosine kinases, and also the phosphatase calcineurin (3, 9). Our experiments using phorbol myristate acetate and staurosporine rule out a significant role for protein kinase C in the control of SERCA expression in VSMC. To confirm the effectiveness of phorbol ester treatment under our conditions, we examined mRNA levels for the plasma membrane Ca\(^{2+}\) pump gene PMCA2 (data not shown). This gene was upregulated in a transient fashion in the VSMC, exactly as observed previously in vascular endothelial cells (18). Further studies with kinase inhibitors were also able to rule out the involvement of Ca\(^{2+}\)/calmodulin-dependent kinases (both KN-62 and staurosporine were without effect) and tyrosine kinases (bis-tyrphostin and genistein were without effect) in the control of SERCA expression.

The Ca\(^{2+}\)-dependent phosphatase calcineurin plays a central role in signaling pathways involved in gene regulation in a number of cell types (8). In the heart, calcineurin activation has been implicated as an early event in the hypertrophic response to cardiac injury (34). Furthermore, calcineurin has also been implicated in the control of both plasma membrane Ca\(^{2+}\) pump and Na\(^+\)/Ca\(^{2+}\) exchanger expression in brain neurons (13, 21). Using the selective and potent calcineurin inhibitor cyclosporine, we were unable to show any effect on the Tg-induced elevation of SERCA expression. Thus it appears that the plasma membrane and sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) transporters are controlled separately and that, in some cases, their expression levels respond in opposite directions to similar stimuli (38).

Despite the number of reagents that were without effect on SERCA expression, we did find that treatment with 50 \(\mu\)M of the nonselective kinase inhibitor, H-7, completely prevented Tg induction of SERCA2b mRNA. Intriguingly, however, 100 nM staurosporine,
another kinase inhibitor with a broad spectrum of specificity, was completely without effect. This suggests the involvement of an H-7-sensitive, but staurosporine-insensitive, protein kinase. Because staurosporine can effectively inhibit protein kinase C, Ca$^{2+}$/calmodulin-dependent kinases, protein kinase A and protein kinase G, among others (39), the target for H-7 inhibition of the Tg effect on SERCA2b mRNA is unclear. Obviously, a novel or less well-characterized protein kinase, of which there are thought to be many (36), is a likely candidate. Unfortunately, however, there are currently few useful pharmacological tools to investigate this issue in a systematic manner. Further delineation of the pathway(s) that controls SERCA gene expression in vascular smooth muscle will, therefore, require more detailed and involved analysis.

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