

Gene amplification and transcriptional upregulation of the sarco/endoplasmic reticulum Ca²⁺ transport ATPase in thapsigargin-resistant hamster smooth muscle cells

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Received March 6, 1998; Revised July 21, 1998; Accepted July 28, 1998

ABSTRACT

We have selected a series of cell lines from the parental Syrian hamster smooth muscle cell line DDT₁-MF₂ that are resistant to thapsigargin (TG), a specific inhibitor of the sarcoplasmic/endoplasmic reticulum Ca²⁺ transport ATPases (SERCAs). Cells were selected for resistance to TG in the presence or absence of cyclosporin (CSA), which is a competitive inhibitor of the multidrug transporter p-glycoprotein (pgp). Since TG is a known substrate for pgp, selection for TG resistance was carried out in the presence of CSA in an attempt to minimize the contribution of pgp, and to identify the potential range of adaptive responses of the SERCA pump itself, during the development of the TG-resistant phenotype. Irrespective of whether the selection is carried out in the presence or absence of CSA, pgp is overexpressed in the TG-resistant DDT₁-MF₂ cells. SERCA protein is also overproduced in the TG-resistant cell lines, which occurs through one of several mechanisms. Included among these, is amplification of the SERCA gene and enhanced transcription of the gene. Enhanced transcription is observed only upon long-term selection and occurs through the SERCA gene proximal promoter elements. Although SERCA transcription in wild-type cells is dependent upon the -284 to -72 bp region of the SERCA promoter, the TG-resistant cells utilize both the -284 to -72 bp and the -72 to +80 bp promoter regions for enhanced SERCA transcription. That is, additional elements within the -72 to +80 bp region are recruited in the TG-resistant cells to allow for increased SERCA expression. A post-transcriptional step may also be recruited by the TG-resistant cells in their overall strategy to produce increased amounts of the SERCA protein. These studies demonstrate that the DDT₁-MF₂ cells can utilize different mechanisms which lead to

increased levels of SERCA protein as the cells adapt to inhibition of the ATPase by TG.

INTRODUCTION

Intracellular Ca²⁺ plays a fundamental role in many cellular processes including proliferation, differentiation and muscle contraction/relaxation. The sarcoplasmic/endoplasmic reticulum Ca²⁺ transport ATPases (SERCAs) are key enzymes involved in the regulation of cytoplasmic Ca²⁺ and intracellular Ca²⁺ stores. They serve as housekeeping enzymes in non-muscle tissues, where their primary function is to accumulate and maintain Ca²⁺ within intracellular stores via ATP-dependent transport. In muscle tissues, SERCAs help reaccumulate Ca²⁺ into the sarcoplasmic reticulum during the relaxation phase of muscle contraction.

Three principle isoenzymes of SERCA (i.e. SERCA1, 2 and 3) have been identified (1–5). The SERCA1 gene encodes two alternatively spliced isoforms, SERCA1a (adult) and SERCA1b (fetal), which are expressed exclusively in fast twitch skeletal muscle (5–7). The SERCA2 gene also encodes two alternatively spliced isoforms: SERCA2a and SERCA2b (1–3). SERCA2a is the predominant isoform expressed in heart and slow twitch skeletal muscle (8), while SERCA2b is expressed in multiple tissues, including smooth muscle and most non-muscle tissues (2,3,9). Two isoforms of SERCA3 have also been identified and are found in endothelial and epithelial cells (9,10).

The sesquiterpene lactone thapsigargin (TG) is a potent and specific inhibitor of the SERCA pumps (11). By inhibiting SERCA function, TG depletes intracellular Ca²⁺ stores, resulting in inhibition of cell proliferation (12). However, high levels of resistance to TG inhibition of cell proliferation can be developed (13,14), which is associated with TG-resistant ATP-dependent Ca²⁺ transport into intracellular stores, as shown *in situ* and following isolation of microsomes from the resistant cells.

In an attempt to identify the potential range of adaptive responses of the SERCA pumps to selective pressure, we have developed a model system based upon the Syrian hamster smooth

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muscle cell line DDT₁-MF₂ in which the major SERCA gene expressed is SERCA2. Thus, we derived, under various selection strategies, a series of cell lines from DDT₁-MF₂ cells that are highly resistant to the inhibitory effects of TG. Previously, we showed that TG is a substrate for the multi-drug transporter p-glycoprotein (pgp) (13). Since TG is a substrate for pgp, to minimize any potential contribution of pgp to the TG-resistant phenotype, we selected a series of cell lines from DDT₁-MF₂ cells for resistance to TG using a strategy that would theoretically minimize for selection of mechanisms of resistance involving pgp. Cyclosporin (CSA) is one of the most potent agents known to reverse pgp-mediated resistance. One would expect that selection of cells for resistance to TG in the presence of CSA should negate or minimize any selective advantage of pgp (although cells with a mutated pgp that is insensitive to CSA could conceivably also survive under these conditions). Therefore, a panel of cell lines were developed by selecting DDT₁-MF₂ cells in increasing concentrations of TG in the absence or presence of CSA.

In this report we demonstrate, for the first time, that several adaptive mechanisms with respect to the SERCA pump become operative in the smooth muscle cells upon development of resistance to TG inhibition, including SERCA2 gene amplification and transcriptional upregulation without concomitant gene amplification. As reported previously for the skeletal muscle cell lines Sol8 and C2C12 (15,16), the -284 to -72 bp proximal SERCA2 promoter region is required for SERCA transcription in the wild-type (wt) DDT₁-MF₂ cells since further promoter deletions are inadequate for transcription in these cells. In contrast, in TG-resistant DDT₁-MF₂ cells, elements within the -72 to +80 bp promoter region appear to cooperate with the -284 to -72 bp promoter region during enhanced SERCA transcription. Thus, during selection for resistance to TG, additional regulatory factors may become activated in the DDT₁-MF₂ cells and be subsequently utilized in SERCA gene transcription. The present study suggests that a certain plasticity in terms of response to selective pressure exists for SERCA, and provides a model to further delineate the mechanisms regulating this class of enzymes.

MATERIALS AND METHODS

Cell lines

The DDT₁-MF₂ cell line was originally established from Syrian hamster vas deferens smooth muscle (17), and obtained from ATCC (Rockville, MD). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 4% enriched calf serum (Gemini Bio-Products, Inc.) plus 50 µg/ml gentamicin (Gibco-BRL). A series of TG-resistant cell lines were derived from DDT₁-MF₂ cells by selecting the latter in sequentially increasing concentrations of TG over the course of several months. At each step of higher drug selection, the majority of cells would die, with a healthy population of cells finally emerging over a period of several weeks in the continued presence of the higher drug concentration. Thus, two TG-resistant sublines, designated DDT/TG500nM and DDT/TG4µM (with the final maintenance concentration of TG being 500 nM and 4 µM, respectively) were derived from the DDT₁-MF₂ cell line by a series of stepwise selections with TG. Of note is that the DDT/TG4µM cell line was derived from DDT/TG500nM cells by further selecting the latter with sequentially increasing amounts of TG. In addition, two other cell lines, designated

DDT/CSA/TG40nM and DDT/CSA/TG500nM, were generated by selecting stepwise DDT₁-MF₂ cells with TG in the presence of 0.2 µg/ml CSA (Sigma) (CSA at 0.2 µg/ml does not inhibit DDT₁-MF₂ cells). After final selection, the DDT/CSA/TG40nM and DDT/CSA/TG500nM cells were maintained in 40 nM TG plus 0.2 µg/ml CSA and 500 nM TG plus 0.2 µg/ml CSA, respectively. Again, of note is that the DDT/CSA/TG500nM cells were derived from the DDT/CSA/TG40nM cell line by further selection of the latter with increasing amounts of TG in the continued presence of 0.2 µg/ml CSA.

Materials for cloning and sequencing

The vector plasmid Bluescript (KS⁺) was purchased from Stratagene Corp. for subcloning and sequencing of the hamster SERCA2 and rat GAPDH cDNA inserts. Amplitaq polymerase and dNTPs were obtained from Perkin-Elmer Cetus (Norwalk, CT). Oligonucleotides for *in vitro* PCR amplification were purchased from Bio-synthesis Inc. (Lewisville, TX). Restriction endonucleases and DNA modification enzymes were purchased from Gibco-BRL (Bethesda, MD) and New England Biolabs (Beverly, MA). Reagents for dideoxy sequencing and ³²P-labelling of the cDNA fragments were purchased from Amersham Corp. (Arlington Heights, IL).

Cloning of cDNA fragments

Approximately 1.0 kb of the 5' end of the hamster SERCA2 cDNA fragment was cloned by PCR. Degenerate oligonucleotides were designed based on the human SERCA2 amino acid sequence (18). The 5' oligonucleotide corresponds to the translation start site amino acid sequence MENAHT [5'-CGCGGATCCATGGAA-(G)AAC(T)GCG(A/T/C)CAC(T)AC-3'; oligo 2.1] while the 3' oligonucleotide is positioned ~330 amino acids downstream from the translation start site and corresponds to the amino acid sequence MAKKNA [5'-CGCGGATCCGCA(G)TTC(T)TTC-(T)TTG(C/A/T)GCCAT-3'; oligo 2.2]. Total RNA from DDT₁-MF₂ cells was reverse-transcribed (RT) using dT₍₁₂₋₁₈₎ primer, and an ~1 kb 5' end cDNA fragment was generated by polymerase chain reaction (PCR) using oligos 2.1 and 2.2 under conditions as detailed previously (19). A 763 bp rat GAPDH cDNA fragment between nucleotide (nt) positions 217 and 979 was generated by RT-PCR using total RNA extracted from rat lung tissue and appropriate oligonucleotide primers (i.e., forward primer: 5'-GG-GGAATTCATCACCATCTTCCAGGAGCG-3' from position 217-236; and reverse primer: 5'-GGGAATTCATGAGGTCC-ACCACCTGTT-3' from position 960-979) (20). The PCR amplified cDNA fragments of hamster SERCA2 and rat GAPDH were cloned into the *Bam*HI and *Eco*RI sites of pBluescript KS⁺. The sequence of the inserts was confirmed via double-stranded dideoxy sequencing of several of the recombinant clones.

Southern and northern blot analysis

Genomic DNA extractions from the various DDT₁-MF₂ cell lines, restriction digestion and Southern blotting were performed according to standard procedures (21). RNA extraction and northern blot analysis were performed essentially as described previously (22). Probe labelling was according to the random primer method of Feinberg and Vogelstein (23). The hybridization and washing conditions were as described by Sambrook *et al.* (21).

Table 1. Characteristics of TG-resistant cells

Cell lines	IC ₅₀ ^a (μ M)	Fold resistance ^b	RNA ^c	Gene copy number ^d	Protein ^e	Relative protein activity ^f
DDT ₁ -MF ₂	0.08	1.0	1.0	1.0	1.0	1.0
DDT/CSA/TG40nM	0.8	10.0	1.5	1.0	–	1.2
DDT/CSA/TG500nM	9.0	112.5	11.5	9.0	20.0	0.8
DDT/TG500nM	1.0	12.5	1.0	–	–	1.4
DDT/TG4 μ M	9.0	112.5	4.0	1.0	8.0	6.0

^aAbsolute IC₅₀ values in μ M.^bRelative resistance to DDT₁-MF₂ cells.Summary of data in ^cFigure 2; ^dFigure 4; ^eFigure 8; ^fFigure 5A.

Western blot analysis

Logarithmically growing DDT₁-MF₂ wt and drug-resistant cell lines were harvested, and cell lysates prepared as described by Gutheil *et al.* (13). SDS–polyacrylamide gels (7% polyacrylamide) were electrophoresed as per the method of Laemmli (24) and transferred onto nitrocellulose membranes. The membranes were probed with SERCA2 antibody (IID8 F6) (25). Similar blots were also probed with the *pgp*-specific monoclonal antibody C219 (26). Bound antibody was detected with a secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase) and a commercially available ECL kit (Amersham Corp.).

Promoter constructs and transient transfections

The rabbit SERCA2 promoter construct p1810-CAT, containing the –1810 to +350 bp region of the SERCA2 gene upstream of the chloramphenicol acetyltransferase reporter gene, has been previously described (15). The –1810 to +350 bp SERCA2 promoter region from p1810-CAT was removed via *Hind*III and *Bam*HI digestion, and cloned between the *Hind*III and *Bgl*II sites of the luciferase reporter plasmid pXP2 to generate p1810-luc. The rabbit SERCA2 promoter constructs p284-luc and p72-luc have been previously described (16). In the p72-luc construct the –72 to +350 bp region of the SERCA gene lies upstream of the luciferase reporter gene (Fig. 7A). The S.1.6 construct was obtained by removing the +80 to +350 bp *Sma*I subfragment from the –72 to +350 bp SERCA gene region present in p72-luc and religating back the remaining plasmid. Thus, in the final S.1.6 plasmid the –72 to +80 bp region of the SERCA gene lies upstream of the luciferase reporter gene (Fig. 7A). The released +80 to +350 bp *Sma*I SERCA exon 1 subfragment was cloned in the *Nru*I site upstream of the CMV promoter in the pCMV-luc vector to generate the S.2.6 plasmid (Fig. 7A). The plasmid pCMV- β was from Clontech Inc. (Palo Alto, CA). This plasmid carries the *Escherichia coli lacZ* gene under the control of the CMV promoter and encodes for β -galactosidase. The promoterless plasmid pXP2-luc (27) was used as a negative control for luciferase activity. Transient transfections were performed using the Ca²⁺ phosphate–DNA coprecipitation method (28). Unless otherwise stated, for transfections in the TG-resistant cells, the cells were plated in 100 mm dishes in the absence of any maintenance TG on the day prior to the transfection. The luciferase and β -galactosidase assays were performed as described previously (29).

RESULTS

Selection of TG-resistant DDT₁-MF₂ cells

The IC₅₀ values of the DDT₁-MF₂-derived cell lines for TG are shown in Table 1. These data reveal that DDT₁-MF₂ cells selected in CSA plus TG acquire the TG-resistant phenotype at relatively lower selection concentrations of TG than those selected in TG alone. For instance, the DDT/CSA/TG40nM cells (selected in CSA plus 40 nM TG) are almost as resistant to TG inhibition as the DDT/TG500nM cells (which are selected only in TG) (Table 1). Furthermore, the DDT/CSA/TG500nM cells are ~9-fold more resistant to TG inhibition than the DDT/TG500nM cells. In fact, the degree of resistance to TG acquired by the DDT/CSA/TG500nM cells is comparable to that achieved when the DDT₁-MF₂ cells are stepwise selected only in TG to high final concentrations (i.e., DDT/TG4 μ M cells) (Table 1). Our original purpose of selecting for TG resistance in the presence of CSA was to optimize for the selection of any potential adaptive responses that might be more specific to the SERCA pump, while minimizing the role of *pgp*, during the acquisition of the TG-resistant phenotype. The above data suggest that selection in the presence of CSA does optimize for the development of TG resistance. It appears, however, that expression of *pgp* is not necessarily modified by CSA when the DDT₁-MF₂ cells are selected for resistance to TG. For instance, both the DDT/CSA/TG500nM and DDT/TG500nM cells overexpress *pgp* to equivalent levels on western blots (Fig. 1).

Expression of SERCA2 in TG-resistant DDT₁-MF₂ cells

When the above cell lines are analyzed with respect to the SERCA pump, several novel observations regarding its response to TG selection are made. SERCA2 protein is well-conserved across species, but due to degeneracy the sequences tend to be more variable at the nucleotide level between species. Complementary DNAs encoding the different isoforms of SERCA have been cloned from many species. However, the hamster species SERCA2 cDNAs have yet to be cloned. To obtain hamster-specific probes for our studies we used 5' and 3' degenerate primers, based on the published rat and human SERCA2 cDNA sequence between nt 1–17 and 976–992, respectively, to clone the 1 kb 5' end of the hamster SERCA2 cDNA. Using this fragment as a probe, RNA samples from the different TG-resistant cell lines were analyzed via northern blots (Fig. 2). Two SERCA2 transcripts of ~4.5 and 8.0 kb size are seen in all the cell lines (Fig. 2A). The above blots were reprobed with a rat GAPDH cDNA fragment

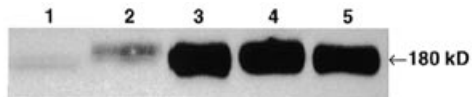


Figure 1. Immunodetection of pgp. Crude membrane fractions were prepared and run on 7% SDS-PAGE gels. After transfer onto nitrocellulose filters, the filters were probed with pgp-specific C219 antibody. Lane 1, DDT₁-MF₂ (40 μ g); lane 2, DDT/CSA/TG40nM (20 μ g); lane 3, DDT/CSA/TG500nM (2 μ g); lane 4, DDT/TG500nM (2 μ g); lane 5, DDT/TG4 μ M (2 μ g).

(Fig. 2B), and SERCA2 expression normalized to the GAPDH RNA signal. These data are presented in Figure 2C and Table 1. As seen in Figure 2, marked overexpression of SERCA2 occurs in the DDT/CSA/TG500nM cells (Fig. 2A, lane 3) which is ~3-fold higher (Fig. 2C) than that seen in the other SERCA2 overexpressor (i.e. DDT/TG4 μ M cells; Fig. 2B, lane 5). Selection at lower drug concentrations does not appear to induce SERCA gene expression. Also of note is that in none of the DDT₁-MF₂-derived cell lines is the skeletal muscle SERCA1 isoform expressed (Fig. 3).

SERCA gene copy number was also evaluated in our panel of TG-resistant cell lines via Southern blot analysis (Fig. 4). Except for the DDT/CSA/TG500nM cells, Southern blots demonstrate that the gene copy number of SERCA is unchanged among the other resistant cell lines when compared to DDT₁-MF₂ parental cells (Fig. 4A). In contrast, a 9-fold increase in SERCA gene copy number occurs in the DDT/CSA/TG500nM cells over that seen in the wt cells (Fig. 4C, Table 1). The level of SERCA mRNA expression (Fig. 2C) is essentially commensurate with the degree of gene amplification (Fig. 4C) in the DDT/CSA/TG500nM cells, suggesting that the overexpression of SERCA in these cells is primarily due to the increase in the gene copy number (see below).

Regulation of SERCA2 gene expression in the TG-resistant cells

To delineate better the mechanisms controlling SERCA expression in the above cells, transient transfections with the SERCA2 promoter-luciferase reporter construct p1810-luc were carried out. In this construct, a region encompassing -1810 to +350 bp of the rabbit SERCA2 promoter is cloned upstream of the luciferase reporter between the *Hind*III and *Bgl*II sites of the plasmid pXP2. As shown in Figure 5A, transfection of p1810-luc into DDT/CSA/TG500nM cells results in luciferase activity which, when normalized to the β -galactosidase activity obtained from the co-transfected pCMV β vector, is essentially the same as the luciferase activity seen in transfected DDT₁-MF₂ cells. This confirms that the marked overexpression of SERCA2 in DDT/CSA/TG500nM cells is due to gene amplification rather than transcriptional upregulation. On the other hand, an ~6-fold increase in luciferase activity is seen upon transfection of p1810-luc into DDT/TG4 μ M cells when compared to DDT₁-MF₂ cells (Fig. 5A). Since Southern blots show no evidence of gene amplification in the DDT/TG4 μ M cells (Fig. 4), this suggests that the increased SERCA expression in these cells is due primarily to an increase in SERCA gene transcription. The rabbit proximal promoter region -284 to +350 bp is sufficient for high level expression in both fast twitch C₂C₁₂ (30) and slow twitch Sol 8 (15) muscle cells. When the p284-luc construct (which contains the -284 to +350 bp proximal promoter upstream

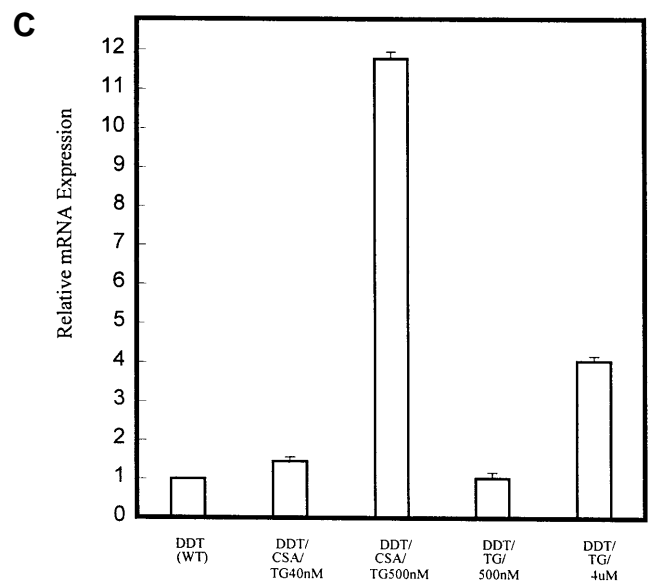
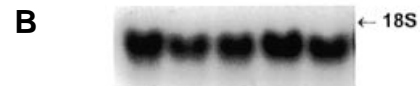
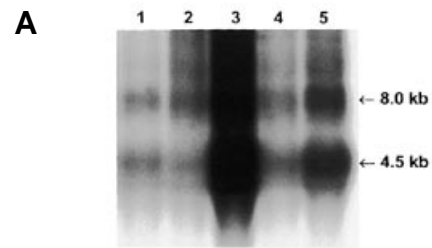


Figure 2. Northern blot of DDT₁-MF₂-derived cell lines. Each lane has 10 μ g of total RNA. (A) Autoradiogram of the blot probed with a hamster SERCA2-specific 5' end cDNA as described in Materials and Methods. (B) Autoradiogram of the blot in (A) probed with rat GAPDH cDNA to assess relative RNA loading in each lane. Lane 1, DDT₁-MF₂; lane 2, DDT/CSA/TG40nM; lane 3, DDT/CSA/TG500nM; lane 4, DDT/TG500nM; lane 5, DDT/TG4 μ M. (C) Histogram showing relative levels of SERCA2 mRNAs in the different cell lines. Columns, mean of two independent experiments; bars, SE.

of the luciferase reporter in pXP2) is transfected into DDT/TG4 μ M cells, a 6-fold increase in luciferase activity over that in parental DDT₁-MF₂ cells is seen (Fig. 5B). This demonstrates that activation of SERCA transcription in DDT/TG4 μ M cells occurs through the proximal promoter elements of the SERCA2 gene. Of note is that, similar to the skeletal muscle cells, the proximal promoter appears to be highly active in the DDT₁-MF₂ cells (compare relative luciferase activity for p1810-luc versus p284-luc in DDT₁-MF₂ cells; Fig. 5B).

The transcriptional upregulation of SERCA in the DDT₁-MF₂ cells occurs only upon long-term selection, and only under specific conditions of selection with TG. For instance, when wt DDT₁-MF₂

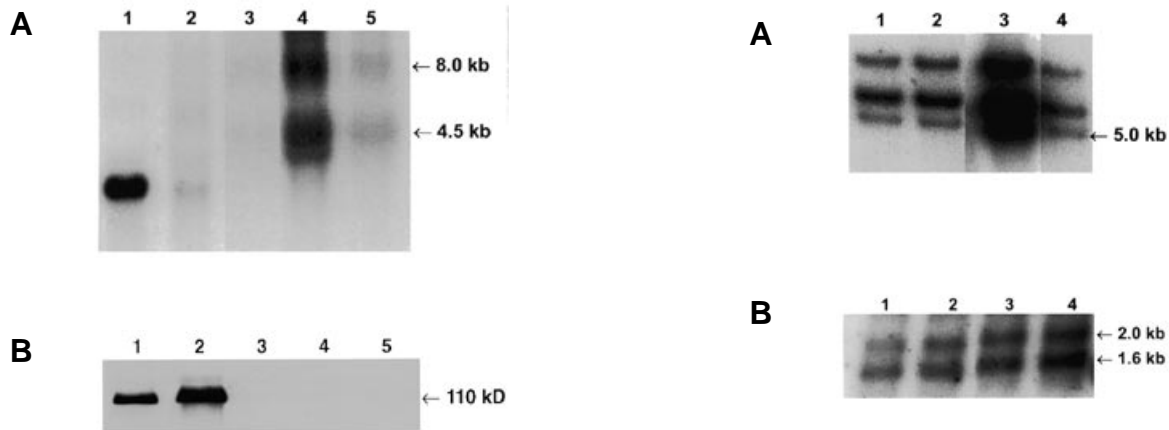


Figure 3. Analysis of SERCA1 expression in DDT₁-MF₂-derived cell lines. (A) Northern blot showing SERCA1 versus SERCA2 expression. When a 5' end SERCA cDNA is used as probe on northern blots it detects the SERCA1 message as a 3.5 kb transcript and the SERCA2 message as 4.5 and 8.0 kb transcripts. That the DDT₁-MF₂-derived cell lines do not express a 3.5 kb message demonstrates SERCA1 is not expressed in these lines. Lanes 1 and 2, skeletal muscle RNA: lane 1, mouse quadriceps muscle; lane 2, rat plantaris muscle. Lanes 3–5, DDT₁-MF₂ smooth muscle RNA: lane 3, DDT₁-MF₂; lane 4, DDT/CSA/TG500nM; lane 5, DDT/TG4 μ M. (B) Western blot. A SERCA1-specific monoclonal antibody (IIIH11; Affinity Bioreagents, Co.) was used to assay for SERCA1 expression. Lanes 1 and 2, hamster quadricep muscle-derived crude cell lysate: lane 1, 5 μ g cell lysate; lane 2, 20 μ g cell lysate. Lanes 3–5, DDT₁-MF₂-derived crude cell lysates (40 μ g of cell lysate per well was used): lane 3, DDT₁-MF₂; lane 4, DDT/CSA/TG500nM; lane 5, DDT/TG4 μ M.

cells are transfected with either the p1810-luc or the p284-luc constructs and treated with TG for 24–48 h, SERCA promoter activity is not induced (Fig. 6A). Although the relative expression of SERCA2 is increased in the DDT/TG4 μ M cells (Fig. 2), SERCA2 promoter activity is not altered significantly in these cells upon transfecting them with SERCA promoter–luciferase reporter constructs followed by a 24–48 h treatment with TG (Fig. 6B). Thus, it is only under long-term selection that certain adaptive responses leading to increased SERCA transcription are induced, and which are not subject to further modulation with short-term manipulations such as those depicted in Figure 6B.

Novel regulation of SERCA2 in DDT/TG4 μ M cells

Previously we showed that although the rabbit SERCA2 proximal promoter region –284 to +350 bp is sufficient for SERCA expression in muscle cells, the –72 to +350 bp promoter region fails to mediate any significant expression of SERCA in these cells (16). The seven Sp1-like elements present between –284 and –72 bp of the SERCA2 promoter were found to be necessary in mediating high level SERCA expression in the muscle cells (15,16). When the p72-luc construct (Fig. 7A) is transfected into wt DDT₁-MF₂ cells, as expected, only low levels of luciferase activity result (Fig. 7B). However, when the same construct is transfected into DDT/TG4 μ M cells, almost a 100-fold increase in luciferase activity is seen in the resistant cells when compared to wt cells (Fig. 7B). These data demonstrate that, in contrast to wt cells, the –72 to +350 bp promoter region becomes highly active in the DDT/TG4 μ M cells.

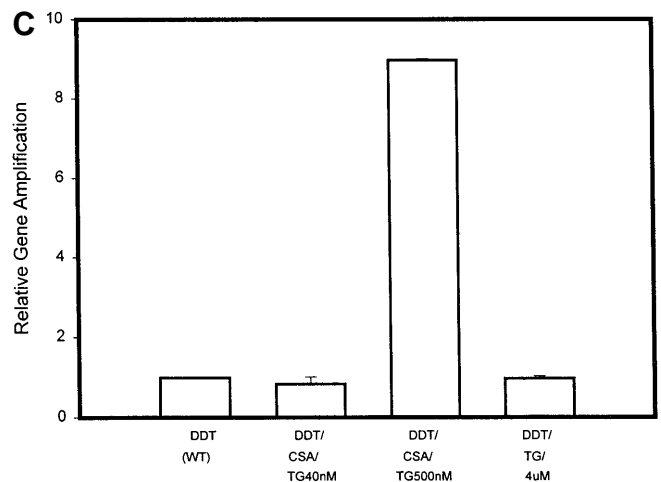


Figure 4. Southern blot of DDT₁-MF₂-derived cell lines. One μ g of genomic DNA was digested with *Hind*III, electrophoresed on 1% agarose gel and the DNA transferred to nitrocellulose filter. (A) Autoradiogram of the genomic DNA Southern blot probed with a hamster SERCA2-specific 5' end cDNA. (B) Same blot reprobed with rat GAPDH cDNA to show relative DNA loading. (C) Histogram derived from the relative signal intensities of the SERCA2-specific 5.0 kb subfragments (obtained upon *Hind*III digestion) present in the different lanes of (A). Lane 1, DDT₁-MF₂; lane 2, DDT/CSA/TG40nM; lane 3, DDT/CSA/TG500nM; lane 4, DDT/TG4 μ M. Columns, mean of two independent Southern blot experiments; bars, SE.

The –72 to +350 bp SERCA2 promoter region was further analyzed by splitting it to generate the S.1.6 and S.2.6 constructs (Fig. 7A). In the S.1.6 construct, the –72 to +80 bp promoter subfragment lies upstream of the luciferase reporter gene, and in the S.2.6 construct the +80 to +350 bp subfragment is cloned upstream of the CMV promoter in the pCMV-luc vector (Fig. 7A). As seen in Figure 7B, the S.1.6 construct is highly active in DDT/TG4 μ M cells when compared to wt DDT₁-MF₂ cells. In contrast, the +80 to +350 bp region does not act as an enhancer upstream of the strong CMV promoter in DDT/TG4 μ M cells (data not shown). That is, only the –72 to +80 bp SERCA2 promoter subfragment becomes activated in the resistant DDT/TG4 μ M cells.

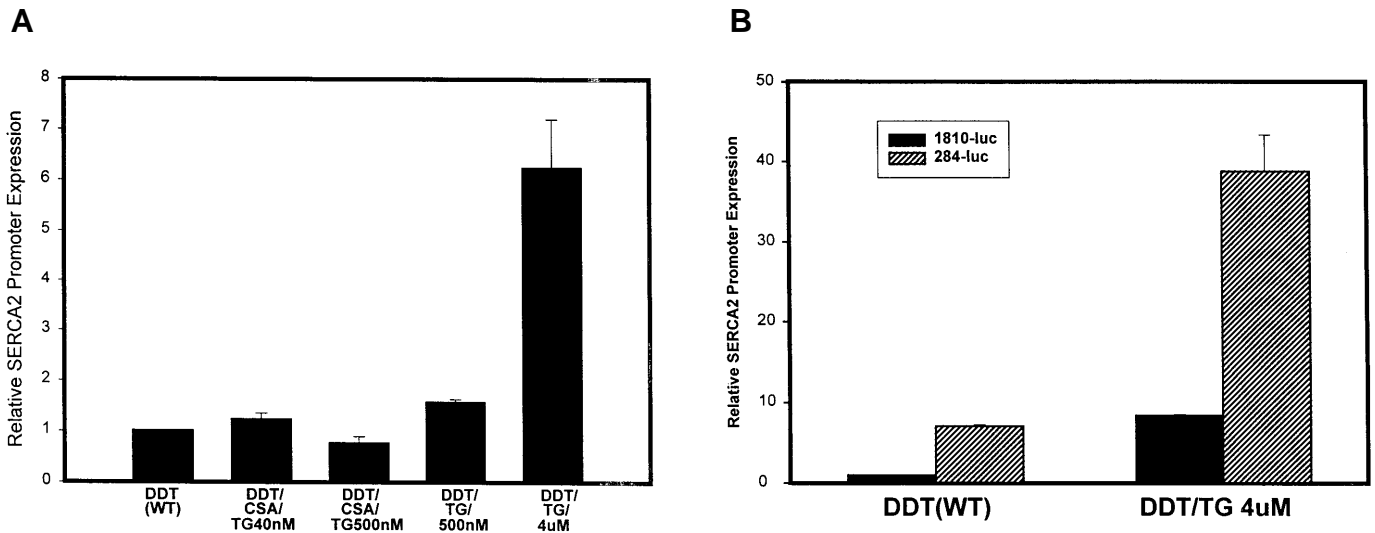


Figure 5. Transient transfections with the SERCA2 promoter-luciferase reporter constructs in resistant cells. For each transfection, 10 μ g of the SERCA2 promoter construct plasmid DNA was co-transfected with 4 μ g of the plasmid pCMV β using calcium phosphate-DNA co-precipitation method. The reported luciferase activities expressed as light units were derived from two independent experiments and normalized to the co-expressed β -galactosidase activities. (A) Histogram of the relative luciferase activities for plasmid p1810-luc transfected into the DDT₁-MF₂-derived cell lines. (B) Histogram of the relative luciferase activities for plasmid p284-luc transfected into DDT₁-MF₂ and DDT/TG4 μ M cells. For comparison, the corresponding relative luciferase activities for plasmid p1810-luc are also shown. Columns, mean of two independent experiments; bars, SE.

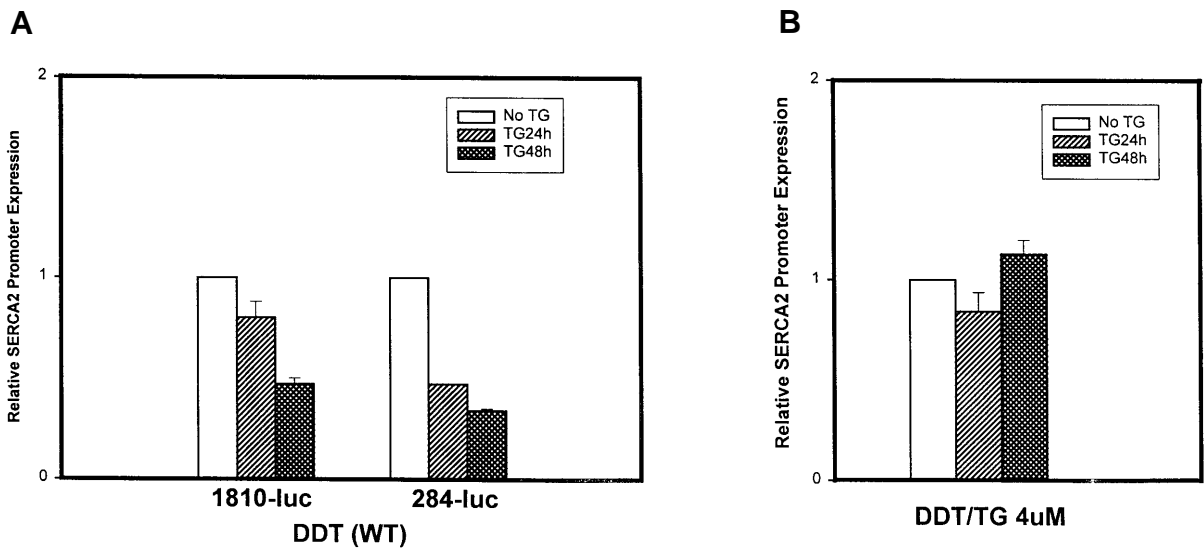


Figure 6. Transient transfections with SERCA promoter-luciferase reporter constructs in wt DDT₁-MF₂ and DDT/TG4 μ M cells. (A) Transfections in DDT₁-MF₂ cells. The plasmids p1810-luc or p284-luc were transfected into DDT₁-MF₂ cells, and the cells harvested 48 h post-transfection for luciferase and β -galactosidase activities. Thus, cells were plated on day 1, transfected on day 2 and harvested on day 4 (i.e. 48 h post-transfection). In control cells no TG was added prior to cell harvest. Other transfected cells were treated with TG (500 nM or 4 μ M) for a period of 24 or 48 h prior to cell harvest. With respect to the 24 h TG treatment, TG was added on day 3 and cells harvested on day 4. For the 48 h treatment, TG was added on day 2 (i.e. on the day of transfection after the cells had been exposed to Ca²⁺ phosphate-DNA co-precipitates for 4 h) and cells harvested on day 4. The data shown here represent treatment with 4 μ M TG for 24 or 48 h. Treatment with 500 nM TG gave essentially identical results (not shown). (B) Transfections in DDT/TG4 μ M cells. p1810-luc was transfected into DDT/TG4 μ M cells, and the cells harvested at 48 h post-transfection. As in (A), some plates were treated with 4 μ M TG for 24 or 48 h, while others (control) were not exposed to any TG prior to cell harvest. Transfection with p284-luc gave similar results (not shown). Columns, mean of two independent experiments; bars, SE.

Although the p72-luc and S.1.6 constructs are active in DDT/TG4 μ M cells (Fig. 7B), a further increase in luciferase activity (~5–7-fold) occurs, when compared to the p72-luc and S.1.6 plasmids, upon transfecting the p284-luc plasmid into these cells (Fig. 7B; note the luciferase activities are represented on a relative log scale). These data suggest that in DDT/TG4 μ M cells

SERCA expression is mediated by *cis* elements located within the –72 to +80 bp and the –284 to –72 bp regions. In wt DDT₁-MF₂ cells, on the other hand, SERCA expression is primarily dependent on elements located within the –284 to –72 bp region since minimal luciferase activity is observed with either the p72-luc or the S.1.6 constructs in the wt cells (Fig. 7B).

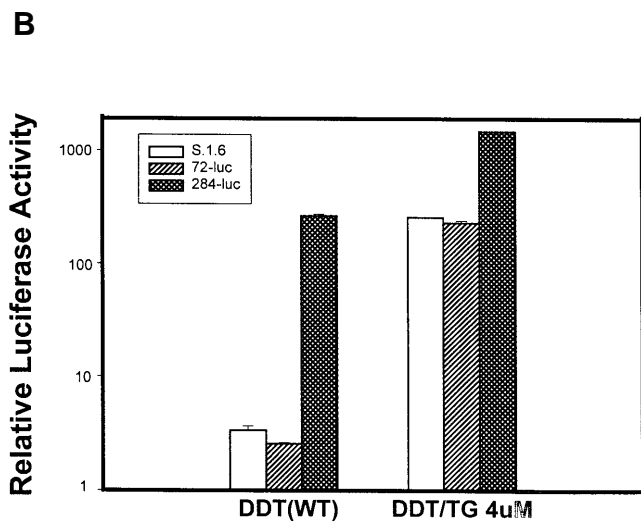
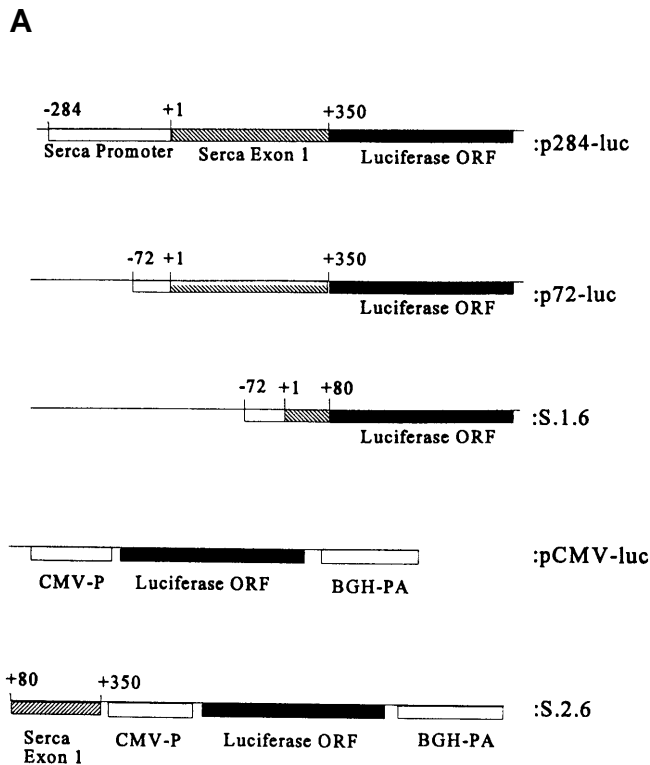


Figure 7. Transfections with SERCA2 proximal promoter constructs in wt and DDT/TG4uM cells. (A) The various SERCA2 proximal promoter constructs are outlined. S.1.6 was derived from p72-luc by removing the +80 to +350 bp SERCA exon 1 region and religating back the remaining plasmid as outlined in Materials and Methods. The S.2.6 plasmid was obtained by cloning the +80 to +350 bp SERCA exon 1 region upstream of the CMV promoter in vector plasmid pCMV-luc. (B) Transient transfections with p284-luc, p72-luc and S.1.6 constructs in wt DDT₁-MF₂ and DDT/TG4uM cells. Note that the luciferase counts are expressed on a log scale. Columns, mean of two independent experiments; bars, SE.

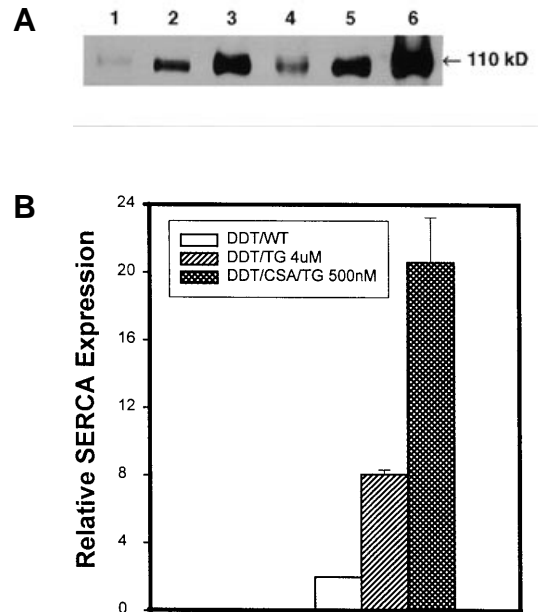


Figure 8. Western blot analysis. (A) Different concentrations of crude cell lysate from the DDT₁-MF₂-derived cells were run on 7% SDS-PAGE gels and analyzed with the SERCA2-specific antibody IID8F6. Lane 1, DDT₁-MF₂ (5 µg); lane 2, DDT/TG4µM (2.5 µg); lane 3, DDT/CSA/TG500nM (2.5 µg); lane 4, DDT₁-MF₂ (10 µg); lane 5, DDT/TG4µM (5 µg); lane 6, DDT/CSA/TG500nM (5 µg). (B) Histogram derived from the relative signal intensities of the SERCA2-specific 110 kDa band in (A). Columns, mean of two independent experiments; bars, SE.

SERCA2 protein in TG-resistant DDT₁-MF₂ cells

The levels of SERCA2 protein as assessed by western blot using whole cell lysates from the DDT/TG4µM and DDT/CSA/TG500nM cells are shown in Figure 8. An 8-fold increase in SERCA2 protein occurs in DDT/TG4µM cells, while a 20-fold increase occurs in DDT/CSA/TG500nM cells when compared to wt DDT₁-MF₂ cells (Fig. 8, Table 1). On the other hand, the relative SERCA2 mRNA level is 4-fold higher in the former and 11.5-fold higher in the latter when compared to the DDT₁-MF₂ cells (Fig. 2, Table 1). Thus, ~2-fold greater accumulation of SERCA2 protein than SERCA2 mRNA occurs in the SERCA2 overexpressing cell lines suggesting that, in addition to gene amplification and transcriptional upregulation, the increased levels of SERCA2 protein in the drug-resistant cells are in part due to activation of a post-transcriptional process(es).

DISCUSSION

Although growth arrest of mammalian cells occurs readily when SERCA function is inhibited by TG, by incremental exposure fibroblasts in culture can be selected so that they continue to proliferate in high concentrations of the ATPase inhibitor (13). Moreover, we have previously shown that the SERCA pumps in the TG-selected cells maintain Ca²⁺ transport function which is highly resistant to inhibition by TG (14). The present study was initiated with the purpose of identifying the potential range of adaptive responses of SERCA upon long-term exposure of cells

to SERCA's specific inhibitor TG. For these studies we employed a model based upon the Syrian hamster smooth muscle cell line DDT₁-MF₂, which has been extensively characterized in terms of Ca²⁺ storage compartments and Ca²⁺ signaling. Although clonal selection may occur upon deriving the TG-resistant cell lines via stepwise selecting the parental cells in sequentially increasing concentrations of drug, the TG-resistant cells in this report were not subcloned prior to further analysis. Hence, the adaptive responses in the TG-resistant cells with respect to both pgp and SERCA are a summation of the responses of the individual cells within each population.

Here we present several novel observations with respect to SERCA in the TG-resistant DDT₁-MF₂ cells. When DDT₁-MF₂ cells are selected for resistance to TG in the presence of a fixed concentration of CSA, marked overexpression of SERCA occurs at the higher levels of TG selection (compare DDT/CSA/TG40nM versus DDT/CSA/TG500nM cells, Fig. 2). Southern analysis reveals that the SERCA gene is amplified in the DDT/CSA/TG500nM cells (Fig. 4). Transfection of an exogenous rabbit SERCA2 promoter (−1810 to +350 bp)-luciferase reporter construct demonstrates that the exogenous promoter is not activated in the DDT/CSA/TG500nM cells when compared to wt DDT₁-MF₂ cells (Fig. 5A). In fact, the luciferase activity is consistently somewhat lower in the former than in the wt cells. Although one cannot say whether each of the amplified genes in the DDT/CSA/TG500nM cells is necessarily transcriptionally active, the fact that the SERCA2 gene is amplified to approximately the same levels to which the SERCA mRNA is expressed, coupled with the transfection data, suggests that SERCA overexpression in these cells is due to gene amplification. The data in Figure 5A also suggest that the presence of multiple SERCA2 gene copies in the DDT/CSA/TG500nM cells do not squelch essential transcription factor(s), thus allowing essentially normal SERCA2 promoter-dependent expression of the transfected luciferase reporter gene.

Increased expression due to gene amplification is a well characterized property of certain growth promoting genes (i.e., oncogenes) during the course of tumor progression. In addition, development of monospecific drug resistance (to antimetabolites like methotrexate) or pleiotropic drug resistance (to polar planar aromatic compounds) is often associated with amplification of the appropriate genes (31–33), particularly in cultured mammalian cells. It is of interest that although TG is a known substrate for pgp, under our selection protocol the gene encoding TG's natural target, i.e. SERCA, and not pgp (data not shown), is amplified. The role of CSA in this process is not clear but the use of a reversal agent of pgp may favor SERCA overexpression through amplification. Although SERCA2 is amplified only in the DDT/CSA/TG500nM cells, events predisposing to gene amplification might become activated at earlier steps of the selection process (for example, in DDT/CSA/TG40nM cells).

In contrast to the DDT/CSA/TG500nM cells, development of TG resistance in DDT/TG4μM cells is associated with a different response in terms of the SERCA gene. No amplification of this gene occurs in the DDT/TG4μM cells (Fig. 4). The increase in SERCA expression in the DDT/TG4μM cells, albeit at lower levels than in the DDT/CSA/TG500nM cells, appears to be due to transcriptional activation of the SERCA gene, as demonstrated by the transfection experiments with p1810-luc into these cells (Fig. 5A). Moreover, it is the proximal promoter region (−284 to

+350) that appears to be preferentially utilized in the transcriptional upregulation of SERCA within the DDT/TG4μM cells (Fig. 5B).

The proximal SERCA2 promoter is highly GC-rich with multiple Sp1 binding sites within the −284 to −72 bp region that are necessary for expression (15). In the wt DDT₁-MF₂ cells SERCA expression is primarily dependent upon the −284 to −72 bp region since further deletions of the SERCA promoter–luciferase reporter constructs (Fig. 7A) precipitously decrease reporter gene activity (Fig. 7B). On the other hand, in the TG-resistant DDT/TG4μM cells both the −284 to −72 bp and −72 to +80 bp regions appear to participate in mediating SERCA expression (Fig. 7B). That is, the overall difference in SERCA expression between DDT/TG4μM cells and wt DDT₁-MF₂ cells could be due to utilization of both the −284 to −72 bp and −72 to +80 bp regions in the former, in contrast to utilization of essentially the −284 to −72 bp region in the wt cells. Thus, the DDT/TG4μM cells appear to utilize additional *cis/trans*-factors within the −72 to +80 bp region to allow for the overall transcriptional upregulation of SERCA in these cells. Rather than activation of positive regulatory factors, conceivably altered utilization of potentially negative regulatory elements, leading to increased SERCA expression, could also occur in the DDT/TG4μM cells. Which of the potential mechanisms, i.e. relief of repression during the TG selection process, or activation of positive *cis*-elements/*trans*-factors, or a combination of the two, contributes to SERCA gene regulation in DDT/TG4μM cells is currently not known. However, these cell lines provide a unique model to help dissect the rather complex regulation of the SERCA gene.

In summary, we demonstrate that DDT₁-MF₂ cells use several strategies to accumulate SERCA2 protein during selection for resistance to TG. In contrast, initial studies suggest that SERCA1 expression is not modulated upon selection of the DDT₁-MF₂ cells for TG resistance (Fig. 3). Since the appropriate hamster-specific probes are not available, we have yet to test the above TG-resistant cells for SERCA3 expression. However, the predominant SERCA gene expressed in DDT₁-MF₂ cells is SERCA2, and hence it is unlikely that SERCA3 expression is modified in these cells during the TG selection process. Interestingly, the contribution of pgp to the overall TG-resistant phenotype does not appear to be affected by whether selection for TG resistance is carried out in the presence or absence of a competitive inhibitor of pgp-mediated drug efflux. However, alterations in SERCA and pgp expression may not completely account for TG resistance, and qualitative changes within SERCA can also occur and contribute to the overall TG-resistant phenotype (34).

ACKNOWLEDGEMENTS

This work was supported in part by a Merit Review Award from the Medical Research Services of the Department of Veterans Affairs (A.H.), V.A. Career Development Award (A.H.), a Grant-in-Aid from the American Heart Association-Maryland Division (A.H.) and NIH PO1HL27867; University of Maryland Institutional Research Grant (A.K.R.); and NIH-HL-52318-04 SCOR on Heart Failure (M.P.).

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