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Studies of Ca^{2+} ATPase (SERCA) Inhibition

Giuseppe Inesi,[1,2] Suming Hua,[1] Cheng Xu,[1] Hailun Ma,[1] Malini Seth,[1] Anand M. Prasad,[1] and Carlota Sumbilla[1]

The Ca^{2+} transport ATPase of intracellular membranes (SERCA) can be inhibited by a series of chemical compounds such as Thapsigargin (TG), 2,5-di(tert-butyl)hydroquinone (DBHQ) and 1,3-dibromo-2,4,6-tris (methyl-isothio-uronium) benzene (Br_2-TITU). These compounds have specific binding sites in the ATPase protein, and different mechanisms of inhibition. On the other hand, SERCA gene silencing offers a convenient and specific method for suppression of SERCA activity in cells. The physiological and pharmacological implications of SERCA inhibition are discussed.

KEY WORDS: SERCA inhibition; thapsigargin (TG); 2,5-di(tert-butyl)hydroquinone (DBHQ); 1,3-dibromo-2,4,6-tris (methyl-isothio-uronium) benzene (Br_2-TITU).

INTRODUCTION

The Sarco- and Endoplasmic Reticulum Ca^{2+} ATPase (SERCA) is an intracellular membrane bound enzyme that utilizes the free energy of ATP to transport Ca^{2+} against a concentration gradient. The physiological role of SERCA is to sequester cytosolic Ca^{2+} into membrane-bound intracellular compartments. The stored Ca^{2+} is in turn released as a general messenger for cellular signaling. Sequencing of cDNA clones (MacLennan *et al*., 1985) has shown various SERCA isoforms that play a fundamental role for Ca^{2+} signaling not only in muscle cells, but also in cells of other tissues. It is apparent that SERCA isoforms are involved in Ca^{2+}-signaling mechanisms for many biological functions, including excitation contraction coupling, excitation secretion coupling, regulatory phosphorylation and dephosphorylation of proteins, gene transcription, intracellular proteolysis and apoptotic mechanisms. In striated muscle, the Ca^{2+} ATPase of sarcoplasmic reticulum (SR) is a particularly abundant protein, and plays a very important role in excitation–contraction coupling. In fact, Ca^{2+} ATPase defects have been detected in experimental and human pathology. Inadequate SR ATPase expression and altered handling of intracellular Ca^{2+} are believed to be an important pathogenetic factor in heart failure (Gwathmey *et al*., 1987).

SERCA belongs to the "P" family of cation transport ATPases, all of them forming a phosphorylated enzyme intermediate by covalent interaction of the ATP terminal phosphate with an aspartyl residue at the catalytic site. The high yield of Ca^{2+} ATPase with the microsomal fraction of muscle homogenates has created uniquely favorable opportunities for functional and structural studies of the native enzyme. The catalytic and transport cycle has been the subject of detailed characterization. Electron microscopy and diffraction studies revealed that the ATPase includes a membrane bound region connected through a stalk to an extramembranous (i.e., cytosolic) headpiece. Furthermore, three-dimensional crystals of native SR ATPase were obtained, yielding high resolution structural models (Toyoshima *et al*., 2000, 2002, 2004; Sorensen *et al*., 2004) of the ATPase molecule in the Ca^{2+} bound state, in a Ca^{2+}-free stabilized state, and under other conditions yielding analogs of various intermediate states of the catalytic cycle.

On the other hand, the availability of cDNA clones has allowed expression of recombinant ATPase in cultured cells (i.e., COS1). Mutational analysis of recombinant

[1] Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Maryland 21201.
[2] To whom correspondence should be addressed; e-mail: ginesi@umaryland.edu.

ATPase has been very useful for evaluation of the role of various residues in the ATPase mechanism. Site directed mutagenesis has provided the original evidence for location of the Ca^{2+}-binding sites within the membrane bound region, location of the catalytic site within the extramembranous headpiece, location of the thapsigargin inhibitory site at the membrane cytosolic interface, and the role of various residues in the functional linkage of Ca^{2+}, catalytic and inhibitory sites. The availability of cDNA clones has also rendered possible gene transfer into cultured cells, and studies of the functional consequences of SERCA overexpression or downregulation in situ.

CHEMICAL (PHARMACOLOGICAL) INHIBITORS

SERCA is responsive to various compounds demonstrating different degrees of potency and inhibition, and permitting studies of the catalytic and transport mechanism, as well as of the consequences of SERCA inhibition on vital cell functions that are dependent on Ca^{2+} signaling. A most potent and specific inhibitor is thapsigargin (TG), a plant-derived sesquiterpene lactone (Rasmussem *et al*., 1978). TG is effective with a stochiometry equivalent to that of the ATPase and a Kd in the sub-nanomolar range (Sagara and Inesi, 1991). The interesting aspect of the TG inhibition is its long range character, involving both phosphorylation and Ca^{2+}-binding sites. In fact, TG forms a dead end complex with the ATPase, with characteristics equal or similar to the ground state (E2). This property turned out to be very useful for stabilization of ATPase crystals obtained in the absence of Ca^{2+}, thereby permitting diffraction studies.

Our laboratory has been involved in chemical synthesis of TG analogs. For instance, we produced a radioactive azido derivative that was used to photolabel the ATPase (Hua and Inesi, 1997), and a dansylated derivative used in fluorescence studies of its interaction with the ATPase (Hua *et al*., 1995). Furthermore, we found that mutations in the S3 stalk segment reduce the affinity of the ATPase for TG (Zhong and Inesi, 1998), and proposed that the S3 stalk segment contributes to the TG inhibitory site, between the membrane-bound and cytosolic regions of the ATPase. Further mutational analysis in parallel with crystallographic data defined the thapsigargin-binding site and identified F256 as an important residue for both binding and inhibitory effect of thapsigargin (Xu *et al*., 2004). This agrees with our previous studies showing that production of resistant cells by selective pressure with thapsigargin in culture, includes selection of spontaneous F256 mutants (Yu *et al*., 1999). TG binds with very high affinity in a cavity delimited by M3, M5, and M7, where Phe256 has an important role in binding and mediating the inhibitory effect. The important fact is that in addition to binding TG by ring stacking, F256 is prevented from undergoing a displacement concomitant with the E2 to E1-2Ca^{2+} transition, and this explains the mechanism of inhibition.

We have also performed studies with 2,5-di(tert-butyl)hydroquinone (DBHQ), another SERCA inhibitor, which appears to bind to a neighboring but distinct site relative to TG in the ATPase molecule. The DBHQ inhibitory mechanism is similar to that of TG, i.e. stabilization (weaker than TG) of the ground state of the enzyme in a dead end complex. Inhibition by DBHQ, on the other hand, is not interfered with by mutation of Phe256, but is rather sensitive to mutation of Leu311 and, to some extent, V62 and D59, suggesting interaction with the M1 and M4 transmembrane helices. From the functional standpoint, both TG and DBHQ stabilize a compact ATPase conformation, and interfere with Ca^{2+} binding and phosphoenzyme formation. Therefore, the functional interference of TG and DBHQ on the ATPase cycle is similar, and their binding sites are in neighboring but distinct locations.

We now find that 1,3-dibromo-2,4,6-tris (methylisothio-uronium) benzene (Br_2-TITU), another ATPase inhibitor (Berman and Karlish, 2003), displays a mechanism quite different from that of TG and DBHQ. Br_2-TITU inhibits ATP hydrolysis and Ca^{2+} transport, but allows the enzyme to bind Ca^{2+} and to undergo phosphorylation by ATP. A most evident effect of Br_2-TITU (Hua *et al*., 2005) is that the level of ADP sensitive phosphoenzyme (i.e., E1P-2Ca^{2+}) observed in the transient state following addition of ATP is much higher in the presence than in the absence of the inhibitor (Fig. 1). Br_2-TITU does not interfere with enzyme phosphorylation by Pi in the reverse direction of the cycle (i.e., E2P), and produces only a slight inhibition of its hydrolytic cleavage. Furthermore, experiments on conformation dependent protection from proteolytic digestion suggest that, under equilibrium conditions, Br_2-TITU does not alter the position of the A domain in the E2, E1-2Ca^{2+} or E2P states. Therefore, the inhibitory effect of Br_2-TITU on steady-state ATPase velocity is attributed to kinetic interference with the E1P-2Ca^{2+} to E2P transition, possibly due to slowing of A domain oscillations and retardation of its positioning from the E1P-2Ca^{2+} to the E2P conformation.

These effects of Br_2-TITU are opposite to those of TG and DBHQ, whose mechanism of inhibition is related to stabilization of a compact ATPase conformation, and interference with Ca^{2+} binding and phosphoenzyme formation. Our experiments with Br_2-TITU provide the first demonstration of a kinetic limit posed by an inhibitor

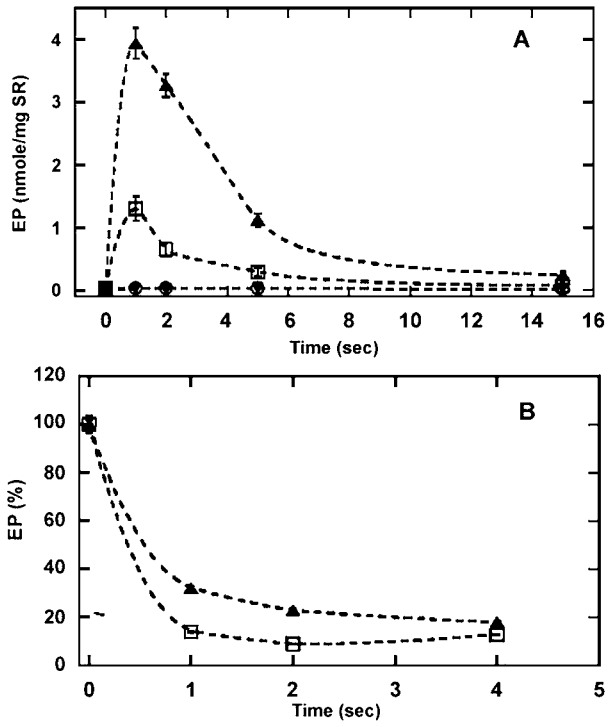

Fig. 1. Enzyme phosphorylation by ATP in the absence (□) and in the presence of 100: μM Br_2-TITU (▲), 6 μM TG (○), and 100: μM DBHQ (▼). A: Time course of phosphoenzyme phosphorylation following addition of ATP. B: Time course of phosphoenzyme decay by addition of ADP and reversal of the pump, demonstrating that the enzyme is in fact in the E1P-2Ca^{2+} state. (From Hua *et al.*, 2005.)

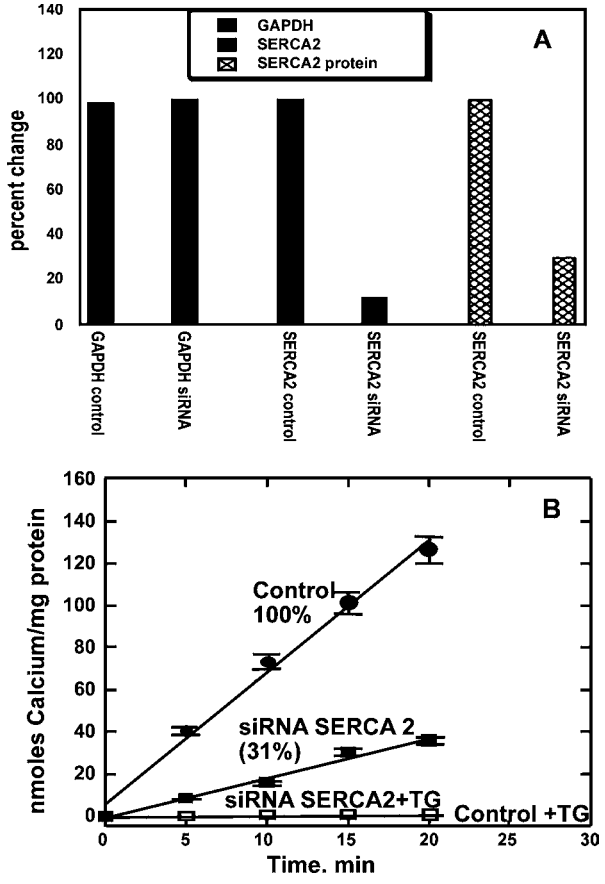

Fig. 2. Effects of SERCA2A gene silencing in cultured cardiac myocytes. A: Reduction of SERCA2A transcript and protein (Western blots). B: Reduction of SERCA operated Ca^{2+} transport by cardiac myocyte homogenates. (From Seth *et al.*, 2004.)

on the E1P-2Ca^{2+} to E2P-2Ca^{2+} transition in the WT enzyme.

SERCA GENE SILENCING

Very useful progress has been recently made in transfer of exogenous cDNA into cultured cells, permitting expression of exogenous SERCA, and characterization of related functional consequences. We were then able to upregulate SERCA levels by expression of exogenous SERCA gene in cardiac myocytes, and demonstrate increased SR ability to sequester Ca^{2+}, and thereby improve cytosolic Ca^{2+}-signaling kinetics and twitch relaxation (Cavagna *et al.*, 2000).

Conversely, we are now able (Seth *et al.*, 2004) to downregulate endogenous SERCA expression to 10–20% of normal level by the use of small interference RNA in cultured cardiac myocytes. RNA interference (RNAi) is a mechanism of posttranscriptional control whereby small double stranded RNA fragments (siRNAs) induce specific degradation of mRNA with complementary sequence by acting as guides for enzymes that cleave the target mRNA (Nykanen *et al.*, 2001). Small interference RNAs can be introduced into cells by classical transfection methods. Limitations of these methods, for our experiments, are related to the requirement for preliminary synthesis, the limited efficiency of transfections (i.e., percentage of cells affected), and the short lifetime. For these reasons we prefer endogenous expression of siRNA through introduction of cDNA templates by adenovirus vectors under control of the U6 promoter, thereby obtaining transcription of fold-back stem-loop structures that yield siRNAs through intracellular processing. The adenovirus vector insures delivery of the cDNA template to all embryonic or neonatal cardiac myocytes. The consequences of this procedure must be monitored by evaluation of transcripts, protein expression and resulting phenotype. In fact, we obtain a very effective and uniform downregulation of SERCA2 expression in all cells undergoing proliferation in culture (Fig. 2), as documented by RT-PCR, Western blotting, as

well as fluorescence microscopy of immunostained cells. Direct measurements of ATP dependent Ca^{2+} transport in cell homogenates also show a prominent reduction. On the other hand, we find that reduction of SERCA2 expression appears to be associated with up regulation of TRP channel (TRPC4 and TRPC5) and Na^{+}/Ca^{2+} exchanger (NCX) protein expression, as shown by RT-PCR as well as by dye assisted or electrophysiological measurements in whole cells (Seth *et al*., 2004). This suggest a possible compensation through transitional crosstalk. However, non-specific effects of adenovirus vectors on transcriptional homeostasis must be carefully evaluated. To this aim, comperison of SERCA sileincing with SERCA inhibition by TG will be most useful. The wide significance of this regulatory mechanism is related to its general involvement in Ca^{2+}-signaling dynamics, as well as in cardiac development and hypertrophy.

PHYSIOLOGICAL AND PHARMACOLOGICAL IMPLICATIONS

Inhibitory compounds, such as TG, have been used extensively to study the dependence of cellular function on Ca^{2+} signaling (reviewed by Treiman *et al*., 1998). TG interference with such important functions as contraction of cardiac muscle (Kirby *et al*., 1992) and proliferation of cultured cells (Ghosh *et al*., 1991) are prominent examples. The therapeutic use of TG aimed at prostate cancer cells is under investigation (Denmeade and Isaacs, 2005). In addition, SERCA gene silencing studies (Seth *et al*., 2004) indicate that components of the Ca^{2+}-signaling mechanism, including the Ca^{2+} ATPase, internal stores and plasmalemmal pathways, may undergo dynamic adjustments through transcriptional crosstalk. In turn, the modality of cytosolic Ca^{2+} elevations plays a role in regulation of gene expression through calcineurin-NFAT-mediated signaling (Molkentin, 2004; Olson and Williams, 2000; Vega *et al*., 2003; Williams and Rosenberg, 2002; Im and Rao, 2004). This appears to be relevant to cardiac development, remodeling and hypertrophy.

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