Role of the S3 Stalk Segment in the Thapsigargin Concentration Dependence of Sarco-endoplasmic Reticulum Ca\textsuperscript{2+} ATPase Inhibition

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The sarco-endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) is specifically inhibited by thapsigargin (TG), whereas the Na\textsuperscript{+},K\textsuperscript{+}-ATPase is not. Large chimeric exchanges between Ca\textsuperscript{2+} and Na\textsuperscript{+},K\textsuperscript{+}-ATPases (Norregaard, A., Vilsen, B., and Andersen, J. P. (1994) J. Biol. Chem. 269, 26598–26601), as well as photocaging with a TG azido derivative (Hua, S., and Inesi, G. (1997) Biochemistry 36, 11865–11872), suggest that the S3-M3 (stalk and membrane-bound) region of the Ca\textsuperscript{2+} ATPase is involved in TG binding. We produced small site-directed changes in the S3 stalk segment of the Ca\textsuperscript{2+} ATPase and found that mutation of five amino acids to the corresponding Na\textsuperscript{+},K\textsuperscript{+}-ATPase residues increases by 3 orders of magnitude the TG concentration required for inhibition of Ca\textsuperscript{2+} ATPase and coupled Ca\textsuperscript{2+} transport. A single mutation in the S3 stalk segment (Glu\textsuperscript{287} → Ile) is sufficient to increase by 1 order of magnitude the TG concentration required to produce 50% inhibition. By comparison, mutations yielding a nine-amino acid homology in the M3 transmembrane segment, or a 25-amino acid homology in the S4 stalk segment, do not affect the ATPase sensitivity to TG. We suggest that specific binding of TG to the S3 stalk segment, in addition to stacking of the TG ring structure at the membrane interface, determines the high affinity of the ATPase for the inhibitor.

The sesquiterpene lactone thapsigargin (TG), isolated from the plant *Thapsia garganica* (3), is a highly specific inhibitor of sarco-endoplasmic reticulum Ca\textsuperscript{2+} ATPases (SERCAs) (4, 5). Kinetic studies indicate that a 1:1 TG-ATPase stoichiometric complex is formed at extremely low concentration of TG (6). The inhibition involves Ca\textsuperscript{2+} binding, formation of phosphorylated intermediate, ATP hydrolysis, and Ca\textsuperscript{2+} transport (7–10). These effects suggest a global effect of TG on the enzyme, as also indicated by its influence on formation of ordered ATPase arrays (11, 12). Hence, clarification of the TG binding domain would contribute to the understanding of structural and mechanistic features in the enzyme. A useful approach, in this regard, is to study the TG sensitivity of chimeric proteins consisting of defined parts of SERCA and Na\textsuperscript{+},K\textsuperscript{+}-ATPase, because TG interacts specifically with the former and not with the latter. Previous studies with large chimeric exchanges, however, produced strong inhibition of catalytic turnover and transport. Nevertheless, Ca\textsuperscript{2+}-dependent formation of phosphorylated intermediate was preserved, and a reduced sensitivity of this parameter to TG was obtained upon chimerization of the entire S3-M3 (stalk and membrane-bound) region (13). In contrast, the TG sensitivity was not altered significantly if other large regions were exchanged (13–15). We describe here the construction and functional characterization of more discrete chimeric changes, involving stepwise mutations of one and up to several amino acids in the S3 or S4 stalk segments of the SERCA, to match the corresponding residues of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase. A similar strategy was previously used in studies of ouabain binding by the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, taking advantage of ouabain-sensitive and ouabain-insensitive isoforms of the enzyme (16).

EXPERIMENTAL PROCEDURES

**PCR Mutagenesis and Protein Expression**—The chicken fast muscle SERCA-1 cDNA (17), containing 11 artificial and unique restriction sites spaced at approximately 300-bp intervals to facilitate cassette exchange (18), was subcloned into pUC19 vector for site-directed mutagenesis. For this purpose, the cassette delimited by BamHII and BssHII restriction sites (579 bp including S3 and M3 coding sequences), and the cassette delimited by BamHII and BssHII (318 bp including M4 and S4 coding sequences), were amplified by PCR using oligonucleotide “flanking” primers. Furthermore, complementary mutagenic oligonucleotides of 23–35 bp length were synthesized for each individual mutation. These primers were utilized to hybridize DNA sequences internal to the flanking primers and were used for PCR mutagenesis by the overlap extension method as described by Ho et al. (19). Briefly, two overlapping fragments containing the mismatched base(s) of the targeted sequence were amplified in separate PCR reactions. The PCR mixtures contained 1 μM each of flanking and mutagenic primers, 800 μM dNTPs, 20 ng of SERCA-1 cDNA, 2.5 units of Pfu DNA polymerase, and Pfu buffer (Stratagene, Menasha, WI) in a final volume of 100 μl. The reaction products were separated by electrophoresis on a 3% low melting agarose gel (FMC, Rockland, ME), and the appropriate M0 band was excised and melted at 72 °C for 5 min. The eluted fragments were fused, and the entire cassette was amplified using both flanking primers. The mutant cassette was then exchanged with the corresponding cassette of wild-type cDNA in pUC19 vector, and sequenced by the dyeoxy chain-termination method using Sequenase (U.S. Biochemical Corp.). Additive chimeric mutations were introduced by sequential PCR mutagenesis using mutant DNA as template. Finally, the mutated cDNA was subcloned into COS-1 expression vector pCDL-SRα296 (20) for transfection and overexpression of protein under control of the SV40 promoter. COS-1 cell cultures and transfections were carried out as described by Sumbilla et al. (15).

**Microsomal Preparation and Immunodetection of Expressed Protein**—The microsomal fraction of transfected COS-1 cells was obtained by differential centrifugation of homogenized cells (15). Immunodetection of expressed ATPase in the microsomal fraction was obtained by Western blotting, using the CaF-5C3 monoclonal antibody to SERCA-1 (17), as described by Sumbilla et al. (15).

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obtained from COS-1 cells transfected with wild-type SERCA-1 cDNA.

The native sequences of the S3 (stalk) and M3 (transmembrane) segments of SERCA-1 (17, 25) and of the corresponding segments of the rat kidney Na\(^+\).K\(^+\)-ATPase α1 isoform (26, 27) are aligned (top and bottom, respectively) according to Norregaard et al. (1). In the S3 segment (A), one out of nine amino acids is identical in the two ATPases, and eight are subjected to stepwise mutation from the Ca\(^{2+}\)- to the Na\(^+\).K\(^+\)-ATPase sequence to yield a nine amino acid segment of homology (Mutant S3,8). In the M3 segment (B), four out of nine amino acids are homologous, and five are mutated from the Ca\(^{2+}\) to the Na\(^+\).K\(^+\)-ATPase sequence to yield a nine amino acid segment of homology (Mutant M3,5).

### Functional Studies

ATP-dependent Ca\(^{2+}\) transport was measured by following the uptake of radioactive calcium tracer by microsomal vesicles. The reaction mixture contained 20 mM MOPS, pH 7, 80 mM KC\(_1\), 5 mM MgCl\(_2\), 0.2 mM CaCl\(_2\), 0.2 mM EGTA, variable concentrations of TG, 5 μg of microsomal protein/ml, 5 mM potassium oxalate, and 3 mM ATP. The reaction was started (37 °C) by the addition of oxalate and ATP, and was terminated at sequential times by vacuum filtration through 321 322 323 324 325 326 327 328 329 330 331 332 333. The filters containing the calcium loaded vesicles were washed with 2 mM LaCl\(_3\) and 10 mM MOPS, pH 7.0, and then shown how eight amino acids of the Ca\(^{2+}\)-dependent ATPase from the total ATPase and was corrected to reflect the level of expressed ATPase in each microsomal preparation, as revealed by immunoreactivity, and with reference to microsomes obtained from COS-1 cells transfected with wild-type SERCA-1 cDNA.

### RESULTS

**Description of Mutants**—The chimerization scheme for analysis of the S3 stalk segment is shown in Table I. In part A (top and bottom lines), nine amino acids of the SERCA S3 segment and of the corresponding Na\(^+\).K\(^+\)-ATPase segment are aligned according to Norregaard et al. (1). In the intervening lines, it is then shown how eight amino acids of the Ca\(^{2+}\)-ATPase sequence were stepwise mutated to complement the conserved Phe\(^{256}\), and yield a nine-amino acid chimeric sequence identical

### Table I

**Mutation schemes for the S3 (A) and M3 (B) segments**

| Sequence no. | A. | B. |
|--------------|----|----|
| 254          | Ca\(^{2+}\) ATPase        | 260 |
| 255          | Mutant S3,2                | 261 |
| 256          | Glu                        | 262 |
| 257          | Phe                        |    |
| 258          | Gly                        |    |
| 259          | Glu                        |    |
| 260          | Glu                        |    |
| 261          | Ser                        |    |
| 262          | Lys                        |    |

**Table II**

**Mutation scheme for the S4 segment**

| Sequence no. | A. | B. |
|--------------|----|----|
| 321          | Ca\(^{2+}\) ATPase        | 337 |
| 322          | Mutant S4,5                | 338 |
| 323          | Gly                        | 339 |
| 324          | Thr                        | 340 |
| 325          | Arg                        | 341 |
| 326          | Arg                        | 342 |
| 327          | Met                        | 343 |
| 328          | Ala                        | 344 |
| 329          | Lys                        | 345 |
| 330          | Asn                        | 346 |
| 331          | Ala                        |    |
| 332          | Lys                        |    |
| 333          | Ser                        |    |

**Table III**

**Effects of single mutations on SERCA-1, and alternative mutations on the S3,5 chimera**

| Original cDNA | Mutation | K\(_i\) Mean ± SD |
|---------------|----------|------------------|
| WT            | Gly\(^{257}\) → Ile | 0.18 ± 0.03 |
| WT            | Gln\(^{296}\) → Leu | 2.22 ± 0.70 |
| S3,5          | Gly\(^{257}\) → Ala | 0.40 ± 0.10 |
| S3,5          | Gln\(^{296}\) → Asn | 200 ± 50  |
| S3,5          | Gly\(^{257}\) → Ala | 0.22 ± 0.07 |
| S3,5          | Gln\(^{296}\) → Gly | 1.50 ± 0.25 |
SERCA-1 enzyme as the catalyst. Note the total inhibition of Ca\textsuperscript{2+} uptake by TG, whereas the ATPase activity displays a component that is Ca\textsuperscript{2+}-independent and TG-insensitive. The symbols refer to: no TG (■), 0.1 nM TG (●), 0.2 nM TG (○), 0.5 nM TG (◇), 10 nM TG (▲), 20 nM TG (△), and no Ca\textsuperscript{2+} (▲).

Fig. 2. Examples of ATP-dependent Ca\textsuperscript{2+} uptake (A) and ATPase activity (B). These steady state measurements were performed as explained under “Experimental Procedures,” using wild-type SERCA-1 enzyme as the catalyst. Note the total inhibition of Ca\textsuperscript{2+} uptake by TG, whereas the ATPase activity displays a component that is Ca\textsuperscript{2+}-independent and TG-insensitive. The symbols refer to: no TG (■), 0.1 nM TG (●), 0.2 nM TG (○), 0.5 nM TG (◇), 10 nM TG (▲), 20 nM TG (△), and no Ca\textsuperscript{2+} (▲).

Fig. 3. Effects of various mutations on the activity of SERCA-1 enzyme expressed in COS-1 cells. cDNA mutations and transfections were carried out as described under “Experimental Procedures.” The microsomal fraction of the transfected cells was obtained by differential centrifugation of homogenized cells, and was utilized for measurements of ATP-dependent Ca\textsuperscript{2+} uptake (see “Experimental Procedures”).

Limited perturbations in this region.

Levels of Expression—Approximately 10% of the COS-1 cells transfected under our conditions overexpress and target the Ca\textsuperscript{2+} ATPase to the endoplasmic reticulum, as shown by in situ microscopic visualization following immunofluorescent staining (18). In the experiments reported here, Western blot analysis of microsomal fractions obtained from the harvested cells revealed similar levels of expression for the wild-type ATPase and ATPase mutants (Fig. 1). Minor variations of expression levels were generally related to the efficiency of transfection rather than the presence of mutations. At any rate, the expression levels were quantitated by densitometry of Western blots, and the resulting values were used to correct the functional parameters to be described below, with reference to the wild-type enzyme.

Ca\textsuperscript{2+} Uptake and ATP Hydrolysis—As originally reported by Maruyama and MacLennan (22), microsomal vesicles obtained from transfected COS-1 cells sustain ATP-dependent Ca\textsuperscript{2+} uptake and related ATPase activity. These are specific and useful functional signals, which, as shown in Fig. 2, proceed at constant rates for several minutes. Ca\textsuperscript{2+} uptake is a highly specific functional parameter, which is totally inhibited by TG (Fig. 2A). On the other hand, the observed ATPase activity includes Ca\textsuperscript{2+}-independent and TG-insensitive components that must be subtracted from the total in order to obtain the specific Ca\textsuperscript{2+}-dependent and TG-sensitive ATPase activity (Fig. 2B).

When we compare the Ca\textsuperscript{2+} transport activities of wild-type and mutant proteins (Fig. 3), we find that the transport rates are unaffected by mutations of up to four amino acids in the S3 segment (mutants S3,2 and S3,3; Fig. 3), but undergo a progressive reduction as the number of mutated amino acids in the S3 segment is increased (mutants S3,4 to S3,8; Fig. 3). On the other hand, the nine-amino acid chimeric homology with the Na\textsuperscript{+},K\textsuperscript{−}-ATPase in M3 (M3,5) and the 25-amino acid homology in S4 (S4,5), produce only 60% and 30% reduction of the transport rates, respectively (see Fig. 3). Similar effects of mutations were observed on the ATPase hydrolytic rates (data not shown). We then tested the TG concentration sensitivity of wild-type enzyme and of mutants retaining at least 50% activity.

It is shown in Fig. 4A that the $K_t$ for Ca\textsuperscript{2+} transport inhibition by TG is gradually shifted by 3 orders of magnitude to higher concentrations (1.7 x 10\textsuperscript{-10} M to 1.25 x 10\textsuperscript{-7} M) as the number of mutated amino acids in the M3 segment is increased.
to five or six, to yield a six-or seven-amino acid homologous segment between Ca\(^{2+}\)-ATPase and Na\(^+\),K\(^+\)-ATPase. Similar results were observed for Ca\(^{2+}\)-dependent ATPase activity (Fig. 4B). Because in the work of Norregaard et al. (1), the ATPase sensitivity to TG was lost following an extensive chimeric replacement involving the entire S3-M3 segment, we produced a nine-amino acid chimeric exchange within the M3 transmembrane segment, to be compared with the analogous chimeric exchange produced in the S3 stalk segment. We found that the chimeric exchange in the M3 segment produced approximately 60% transport inhibition (Fig. 3), but had no effect on the ATPase sensitivity to TG (Fig. 5).

To test the specificity of mutations in the S3 segment with regard to TG sensitivity, we then studied a five-amino acid chimeric mutation in the S4 segment of the ATPase (S4,5 in Table II). It should be pointed out that the segment chosen for our studies contains already 16 homologous, and four conservatively replaced, amino acids, when compared with the corresponding segment of the Na\(^+\),K\(^+\)-ATPase (Table II). Therefore, mutation of five heterologous amino acids results in a 21-amino acid chimeric segment. It is of interest that this S4 mutant produces only 30% inhibition of function (Fig. 4), and its TG sensitivity is identical to that of the wild-type enzyme (Fig. 5).

Considering that a five-amino acid mutation in the S3 stalk segment reduces the ATPase sensitivity to TG by 3 orders of magnitude, we then produced more discrete mutations to test the effects of limited perturbations in this region. We found that a single mutation of Gly257 to the corresponding Na\(^+\),K\(^+\)-ATPase residue (Ile) is sufficient, by itself, to reduce the ATPase sensitivity to TG by 1 order of magnitude (Table III). The important role of Gly257 is also revealed by the significant reversal of TG sensitivity reduction observed when the Gly 257\(\rightarrow\)Ile mutation in the S3,5 chimera is changed to the more conservative Gly257\(\rightarrow\)Ala mutation. Additional reversal to WT behavior is produced by a further change involving the Gln259\(\rightarrow\)Leu mutation to the more conservative Gly259\(\rightarrow\)Asn mutation (Table III). This further reversal is not obtained if Glu259 is mutated to Gly (Table III).

**DISCUSSION**

TG produces global inhibition (i.e. phosphoenzyme formation, hydrolytic activity, and Ca\(^{2+}\) transport) of ATPase, whereas no inhibition is produced by TG on the Na\(^+\),K\(^+\)-ATPase (5). This specificity has motivated chimeric studies to obtain information on the TG binding site, assuming that replacement of a critical SERCA sequence with the corresponding Na\(^+\),K\(^+\)-ATPase sequence would interfere with inhibition. Large chimeric exchanges produce nearly total inhibition of hydrolytic activity and Ca\(^{2+}\) transport. Nevertheless, forma-
The ability to sustain Ca\(^{2+}\) transport and hydrolytic activity, and thus ATPase sensitivity to TG, was studied in a series of SERCA mutants with regard to their stepwise site-directed mutations in the S3 stalk segment and with corresponding Na\(^{+}\)K\(^{+}\)-ATPase constructs (1). Diagrams A–E from the studies reported here. Diagram F was derived from Hua and Inesi (2).

In our experiments, we produced small chimeric changes by stepwise site-directed mutations in the S3 stalk segment and studied the so-derived SERCA mutants with regard to their ability to sustain Ca\(^{2+}\) transport and hydrolytic activity, and their sensitivity to TG. Mutations of up to six amino acids in the S3 stalk segment yield an enzyme retaining ample Ca\(^{2+}\) transport and ATPase activities for studies of inhibition by TG. We then found that small chimeric mutations of the S3 segment (Fig. 6 and Table IV) produce a marked reduction of the ATPase sensitivity to TG. Single mutation of Gly\(^{257}\) → Ile is, by itself, very effective. It should be noted that, given higher TG concentrations, the full inhibitory effect is obtained in all cases. This indicates that chimeric mutations in the S3 segment reduce the ATPase affinity for TG, but do not interfere with the inhibitory mechanism.

It is of interest that the SERCA S4 segment includes already, in its native structure, a very high and unique degree of structural homology with the Na\(^{+}\)K\(^{+}\)-ATPase as well as other cation ATPases. Such a localized homology suggests that this region, and the cation binding site in the transmembrane M3 segment are also ineffective.

Our experiments demonstrate that the S3 stalk segment is specifically involved in determining the ATPase sensitivity to TG and suggest that the S3 segment is involved in TG binding. That the reduced sensitivity may be due to long range effects of the S3 mutations is unlikely, because extensive chimeric exchanges in other regions (Fig. 6 and Table IV) do not affect the ATPase sensitivity to TG (1, 15). It is possible that specific interaction of TG with the S3 segment, in addition to stacking of the TG ring structure at the membrane interface (24), determines the high affinity of the inhibitor for the ATPase.

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