Interference with Phosphoenzyme Isomerization and Inhibition of the Sarco-endoplasmic Reticulum Ca\(^{2+}\) ATPase by 1,3-Dibromo-2,4,6-tris(methylisothiouronium) Benzene*

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ATP hydrolysis and Ca\(^{2+}\) transport by the sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) is inhibited by 1,3-dibromo-2,4,6-tris(methylisothiouronium) benzene (Br\(_2\)-TITU) at the micromolar range (Berman, M. C., and Karlish, S. J. (2003) Biochemistry 42, 3556–3566). In a study of the mechanism of inhibition, we found that Br\(_2\)-TITU allows the enzyme to bind Ca\(^{2+}\) and undergo phosphorylation by ATP. 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. Br\(_2\)-TITU does not interfere with enzyme phosphorylation by P\(_i\) in the reverse direction of the cycle (i.e. E2P) and produces only a slight inhibition of its hydrolytic cleavage. The inhibitory effect of Br\(_2\)-TITU on steady state ATPase velocity is attributed to interference with the E1P-2Ca\(^{2+}\) to E2P-2Ca\(^{2+}\) transition. In fact, experiments on conformation-dependent protection from proteolytic digestion suggest that, in the presence of Br\(_2\)-TITU, the loops connecting the “A” domain to the ATPase transmembrane region undergo greater fluctuation than expected in the E2 and E2P states. Optimal stability of the gathered headpiece domains is thereby prevented. These effects are opposite to those of thapsigargin, in which the mechanism of inhibition is related to stabilization of a highly 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 on the E1P-2Ca\(^{2+}\) to E2P-2Ca\(^{2+}\) transition in the wild-type enzyme.

The sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA)\(^3\) is a membrane-bound 100-kDa protein (2) that sustains active transport of Ca\(^{2+}\), coupled to utilization of ATP. The catalytic and transport cycle includes a number of sequential steps (3) beginning with activation of the enzyme ground state (E2) by high affinity binding of 2 Ca\(^{2+}\) (E1–2Ca\(^{2+}\)) on one side of the membrane followed by utilization of ATP to form of a phosphorlated enzyme intermediate (ADP-E1P-2Ca\(^{2+}\)). The free energy derived from ATP is then utilized for isomerization of E1P-2Ca\(^{2+}\) to E2P-2Ca\(^{2+}\), whereby the bound Ca\(^{2+}\) dissociates with lower affinity on the opposite side of the membrane. Finally, the cycle is completed by hydrolytic cleavage of P\(_i\) from E2P (Scheme 1).

Changes of protein conformation, including separation, rotation, and gathering of the three (N, P, and A) cytosolic domains as well as the displacement of transmembrane segments, occur in concomitance with the sequential steps of the ATPase cycle and play an essential role in the mechanism of energy transduction (4–8). In fact, SERCA inhibition can be produced by stabilization of specific conformational states. For example, thapsigargin (TG) binds to the ATPase with high affinity, yielding a dead end complex with the enzyme in ground state (9, 10). We report here a series of experiments on the characterization of the inhibitory mechanism of Br\(_2\)-TITU (1) and its comparative features relative to other SERCA inhibitors such as TG and 2,5-di(tert-butyl)hydroquinone (DBHQ).

MATERIALS AND METHODS

Sarcoplasmic reticulum (SR) vesicles were obtained from rabbit skeletal muscle (11). Br\(_2\)-TITU (Fig. 1) was synthesized according to Tal and Karlish (12). [\(^{32}\)P]ATP and [\(^{32}\)P]Pi were purchased from PerkinElmer Life Sciences. Thapsigargin, ATP, and other reagents were obtained from Sigma.

ATPase hydrolytic activity was determined following P\(_i\) production by a colorimetric method (13). The reaction mixtures contained 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 0.2 mM EGTA, 50 µM CaCl\(_2\), 3 µg of SR protein/ml, 5 µM A23187 ionophore, and 3 mM ATP. The incubation temperature was 25 °C. Ca\(^{2+}\) binding in the absence of ATP was measured by incubating SR vesicles (40 µg/ml) in a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 0.2 mM EGTA, and 400 µM CaCl\(_2\) to yield various concentrations of free Ca\(^{2+}\) and 5 µM A23187 ionophore. TG (1 µM) was added to half of the samples to provide controls exhibiting no specific Ca\(^{2+}\) binding because it was demonstrated previously that TG prevents specific Ca\(^{2+}\) binding (9, 10). The reaction temperature was 25 °C.

Enzyme phosphorylation by ATP was measured in an ice-cold reaction mixture containing 50 mM MOPS, pH 7.0, 80 mM KCl, 2 mM MgCl\(_2\), 50 µM CaCl\(_2\), 1 mg of SR protein/ml, and 5 µM A23187 ionophore. Individual samples (0.2 ml) were started by the addition of 10 µM [\(^{32}\)P]ATP and quenched at various times with 1 µl perchloric acid.

Enzyme phosphorylation with Pi was measured at 25 °C in a reaction mixture containing 50 mM MES, pH 6.2, 10 mM MgCl\(_2\), 2 mM EGTA, various concentrations of [\(^{32}\)P]Pi, and 1 mg of SR protein/ml in a total volume of 0.2 ml. The reaction was acid-quenched after 10 min of incubation by the addition of 1 µl perchloric acid.

For all phosphoenzyme measurements, the quenched reaction mixtures were transferred into ice, and 1 mg of bovine serum albumin/mg microsomal protein was added as carrier. The samples were then washed by repeated centrifugations and resuspensions in 0.125 M perchloric acid and finally dissolved in 1% SDS for determination of radioactivity and residual protein.

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‡ The abbreviations used are: SERCA, sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase; MOPS, 4-morpholinepropane sulfonic acid; MES, 2-N-morpholinoethanesulfonic acid; Br\(_2\)-TITU, 1,3-dibromo-2,4,6-tris-(methylisothiouronium) benzene; TG, thapsigargin; DBHQ, 2,5-di(tert-butyl)hydroquinone; SR, sarcoplasmic reticulum; TNP-AMP, 2(3′-O-(2,4,6-trinitrophenyl)adenosine 5′-monophosphate.
Limited proteolytic digestion was performed in reaction mixtures containing 50 mM MOPS, pH 7.0, 50 mM NaCl, 0.6 mg of microsomal protein/ml and 0.02–0.04 mg of proteinase K/ml or 0.02 mg of trypsin/ml. CaCl2, EGTA, TG, or Br2-TITU were added as indicated in the figures. Following incubation at 25 °C for various time intervals up to 60 min, the reaction was quenched with trichloroacetic acid (2.5%), and the protein was solubilized with SDS (1%), MOPS (0.312 M), pH 6.8, sucrose (3.75%), β-mercaptoethanol (1.25 mM), and bromphenol blue (0.025%). The samples were then subjected to electrophoretic analysis (14) on 12% gels followed by staining with Coomassie Blue. In some cases, Western blots were obtained with the monoclonal antibody MA3-911 (Affinity Bioreagents) followed by goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies and visualization with an enhanced chemiluminescence-linked detection system (Amersham Biosciences).

RESULTS

In agreement with Berman and Karlish (1), we found that steady state Ca2+ ATPase activity is inhibited by Br2-TITU within the micromolar concentration range, yielding a KI of 20 μM (Fig. 2). As opposed to the total inhibition produced by TG (9, 10), inhibition by Br2-TITU is not total but reduces the steady state ATPase activity by ~80%.

Although the previous study of the mechanism of Br2-TITU was done by following the development of TNP-AMP superfluorescence upon enzyme activation (1), we directly characterized the ATPase partial reactions by measurements of radioactive isotopes. To this aim, we first measured Ca2+ binding (reaction 1 in Scheme I) in the absence of ATP. We found that Ca2+ binding is not inhibited by Br2-TITU, whereas total inhibition is obtained with TG and DBHQ (Fig. 3).

When ATP is added to the enzyme activated by Ca2+, a phosphoenzyme intermediate is formed rapidly and reaches a level, which is dependent on the rates of phosphoryl transfer from ATP and subsequent hydrolytic cleavage. The experiments shown in Fig. 4 were performed with leaky vesicles (because of the addition of a Ca2+ ionophore) to prevent Ca2+ accumulation in the lumen of the vesicles and consequent inhibition of phosphoenzyme cleavage. Under these conditions, we observed an early rise of the phosphoenzyme followed by reduction to a lower level as the ATP added (10 μM) to the reaction mixture was exhausted. Interestingly, we noted that in the presence of Br2-TITU the early rise of the phosphoenzyme reached a much higher level and decayed at a slower rate (Fig. 4A). On the other hand, if we added excess ADP 1 s after ATP (i.e. to the peak level of the phosphoenzyme), we observed a rapid decay of the phosphoenzyme both in the absence and in the presence of Br2-TITU (Fig. 4B). This indicates that the phosphoenzyme accumulated in the presence of Br2-TITU is ADP-sensitive and, therefore, still in the E1P-2Ca2+ state. It is noteworthy that no phosphoenzyme formation with ATP was obtained in the presence of either TG or DBHQ (Fig. 4C).

We then tested the effect of Br2-TITU on enzyme phosphorylation by P, in the absence of Ca2+, which yields equilibrium levels of phosphoenzyme through reactions 7 and 6 in the reverse direction of the cycle (Scheme I). We found no inhibition by Br2-TITU, whereas the reaction was strongly inhibited by TG and DBHQ (Fig. 5A). In fact, the phosphoenzyme levels were increased by Br2-TITU when low concentrations of P, were used in the reaction mixture, indicating increased affinity of the enzyme for P, in the presence of Br2-TITU. When we
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Phosphoenzyme formation was measured at 25 °C in a reaction mixture containing 50 mM MES, pH 6.2, 20 mM MgCl₂, 1 mM [³²P]Pi, and 1 mg of SR protein/ml in the presence of 2 mM EGTA or 0.1 mM CaCl₂. Ca²⁺ binding was measured by incubating SR vesicles (40 μg/ml) in a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl₂, 5 μM A23187 ionophore, and 0.1 mM [⁵⁴Ca]CaCl₂, in the absence or in the presence of 1 mM P₃. The concentrations of Br₂-TITU, TG, and DBHQ were 100, 5, and 100 μM, respectively. The experimental values are given in nmol/mg microsomal protein, and the standard deviations are in parentheses.

|          | Control | Br₂-TITU | TG | DBHQ |
|----------|---------|----------|----|------|
| P₁(EGTA) | 2.0 (0.1)| 2.5 (0.15)| 0  | 0    |
| P₁ and Ca²⁺| 0.5 (0.05)| 1.5 (0.1)| 0  | 0    |

It is noteworthy that in the presence of Br₂-TITU, Ca²⁺ binding and enzyme phosphorylation with P₃ were still inhibited by TG or DBH (not shown). Therefore, Br₂-TITU does not interfere with the binding of these inhibitors and their effects. Furthermore, we found that Br₂-TITU had the unexpected effect of partially reversing the Ca²⁺ inhibition of enzyme phosphorylation with P₃. Furthermore, Ca²⁺ binding was reduced in proportion to the enhancement of enzyme phosphorylation (Table I), indicating that the phosphoenzyme formed is in the low Ca²⁺ affinity state (i.e. E₂P).

The kinetic studies described above indicate that the ATPase steady state velocity is inhibited by Br₂-TITU by interference with an isomeric transition of the phosphorylated enzyme intermediate, whereby conversion of E₁P-2Ca²⁺ to E₂P-2Ca²⁺ is delayed. We then performed experiments on limited ATPase protein digestion with proteinase K and trypsin to explore ligand-dependent conformational states of the enzyme. In these experiments we tested the accessibility of proteinase K (Leu-119 and Thr-242) digestion sites. These sites reside on the loops connecting the “A” domain to transmembrane segments M2 and M3 and on the outer loop of the A domain, respectively. The accessibility of these sites, or lack thereof, reflects A domain rotation in concomitance with sequential reactions of the catalytic cycle and corresponds to dissociation or gathering of the ATPase headpiece domains (15, 16).

It is shown in Fig. 6 that, in the absence of Ca²⁺, digestion by proteinase K occurs with a specific pattern (17) yielding a 95-kDa band (cleavage at Leu-119) and an 83-kDa band (cleavage at Thr-242). This pattern of rather slow digestion is attributed to the compact conformation of the ATPase headpiece in the absence of Ca²⁺ (E₂) and the consequent protection of A domain proteolytic sites. When the digestion is performed in the presence of Br₂-TITU, the 95-kDa band is hardly noted, indicating that the Thr-242 site in the A domain is not protected, and the 95-kDa fragment is rapidly cut to yield the 83-kDa product. The opposite is observed in the presence of TG, as a prominent 95-kDa band indicates very efficient protection of the Thr-242 site.

The ATPase digestion with proteinase K proceeds faster in the presence of Ca²⁺, and the 95-kDa band is not present either in the absence or in the presence of Br₂-TITU (Fig. 6). This is attributed to a Ca²⁺-dependent, open conformation of the ATPase headpiece (i.e. E₁–2Ca²⁺) whereby the proteolytic sites of the A domain are exposed (4). It is noteworthy that under
these conditions the digestion is slower in the presence of TG, with a prominent 95-kDa band (Fig. 6). These experiments indicate that Br₂-TITU has no apparent influence on the open conformation of the ATPase headpiece, which is induced by Ca²⁺/H₁¹₀₀₁ (i.e. E₁⁻²Ca²⁺/H₁¹₀₀₁). On the contrary, TG produces stabilization of the E₂ conformation and places a strong limit on the fluctuations of the A domain loops.

Protection from proteinase K is also observed in the presence of Pi and absence of Ca²⁺/H₁¹₀₀₁ (i.e. E₂P). In this case the protection is even higher than in E₂, as the ATPase band is reduced at a lower rate, and the two digestion bands are retained for a longer time (Fig. 7). A distinctive feature of the pattern obtained in the presence of Br₂-TITU is the lack or low intensity of the 98-kDa band (Fig. 7), indicating that the Thr-242 site is not protected. Considering that Br₂-TITU allows (and even favors, at low Pi concentrations) the phosphorylation reaction, it is apparent that considerable fluctuations of the A domain loops are still permitted when the enzyme is phosphorylated by Pi in the presence of Br₂-TITU. On the other hand, the digestion pattern observed in the presence of TG (Fig. 7) is very similar to that observed in the absence of Pi, consistent with interference with the Pi reaction and stabilization of the E₂ state.

Further experiments on proteolytic digestion were performed with trypsin. Trypsin cleaves the ATPase at a first site (T₁, Arg-505 on the N domain), yielding two fragments of nearly equal size (A and B), and then at a second site (T₂, Arg-198 on the outer loop of the A domain), yielding two subfragments of quite different size (A₁ and A₂). Digestion at T₁ is not influenced significantly by the presence of Ca²⁺ or Pi, whereas digestion at T₂ occurs at a rapid rate in the presence of Ca²⁺, as revealed by appearance of the A₁ band in electrophoretic gels (Fig. 8). Digestion at T2 occurs much more slowly in the absence of Ca²⁺ and even more slowly under conditions of enzyme phosphorylation by Pi (15, 16). We found that Br₂-TITU does not change the patterns of digestion observed in the absence or presence of Ca²⁺ and Pi (Fig. 8). Therefore, Br₂-TITU does not interfere with conformation-dependent protection of T2 on the outer loop of the A domain.

DISCUSSION

An inhibitory effect of Br₂-TITU on SERCA was first reported by Berman and Karlish (1), who performed a detailed
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study of fluorescence effects. They observed that Br2-TITU quenches the intrinsic tryptophan fluorescence of the ATPase protein but increases the fluorescence acquired by bound TNP-AMP upon enzyme phosphorylation. These effects were attributed to inhibition of phosphoenzyme hydrolytic cleavage and increased levels of the phosphoenzyme in the E2P state (1).

We confirmed that Br2-TITU inhibits steady state ATPase activity within the micromolar concentration range with a $K_i$ of 20 $\mu$M. To characterize the mechanism of inhibition, we then directly measured Ca$^{2+}$ binding in the absence of ATP, and found that Br2-TITU had no effect on either the affinity or the stoichiometry of binding. Furthermore, phosphoenzyme formation by utilization of ATP in the presence of Ca$^{2+}$ proceeds very efficiently in the presence of Br2-TITU, and much higher levels of ADP-sensitive phosphoenzyme are reached in the presence of Br2-TITU. We also demonstrated by direct measurements that phosphoenzyme formation by utilization of P$_i$ in the absence of Ca$^{2+}$ (i.e. E2P) is not inhibited by Br2-TITU but is in fact favored when the P$_i$ concentration is limiting. This latter effect may be due to loosened A and P domain interaction (see below) and greater accessibility of the phosphorylation site to P$_i$. Hydrolytic cleavage of E2P is only slightly inhibited by Br2-TITU.

The inhibitory effect of Br2-TITU is quite different from that of TG and DBHQ, inasmuch as these inhibitors interfere with Ca$^{2+}$ binding and enzyme phosphorylation with ATP or P$_i$. It is known that TG produces a true dead-end complex, stabilizing a conformational transition equal or quite similar to E2 (10). Thereby, conversion to the E1–2Ca$^{2+}$ conformation in the forward direction of the cycle or to the E2P conformation in the reverse direction of the cycle is precluded by TG binding. On the contrary, Br2-TITU does not produce any stabilization of E2 because we found no hindrance to conversion either to E1Ca$_2$ when Ca$^{2+}$ was added or to E2P when P$_i$ was added in the absence of Ca$^{2+}$. However, under conditions of ATP utilization in the presence of Ca$^{2+}$, the inhibitory effect of Br2-TITU was realized by interference with the E1P-2Ca$^{2+}$ to E2P-2Ca$^{2+}$ transition, resulting in accumulation of E1P-2Ca$^{2+}$. Thereby, formation of E2P was reduced. These direct measurements contradict previous interference based on fluorescence measurements (1), which suggested that Br2-TITU inhibition results in accumulation of E2P by inhibiting its hydrolytic cleavage and is not likely related to interference with the E1P-2Ca$^{2+}$ to E2P-2Ca$^{2+}$ transition.

The experiments on proteinase K and trypsin digestion are indicative of A domain rotation and gathering of the ATPase cytosolic domains following removal of Ca$^{2+}$ to yield E2 (15, 16). Further inclination of the A domain and compact gathering of the headpiece were observed upon addition of P$_i$ to yield E2P. We show here that Br2-TITU interferes significantly with positioning of the A domain and protection of the protease K digestion sites as expected in the E2 and E2P states of the ATPase. It is then apparent that in the presence of Br2-TITU, considerable fluctuation of the loops connecting the A domain to the transmembrane region is allowed even when the enzyme is placed in the E2 or E2P states by equilibration with EGTA or P$_i$. Considering the involvement of A domain positioning and interactions with the P domain in specific phosphoenzyme states (6, 7), we suggest that the Br2-TITU conformational interference is related to the delay of the E1P-2Ca$^{2+}$ to E2P-2Ca$^{2+}$ transition observed in the kinetic experiments. This interference results in accumulation of E1P-2Ca$^{2+}$ and reduction of the steady state velocity of catalytic and transport activity. Therefore, the Br2-TITU inhibition mechanism is quite different from that of TG, which is based on strong stabilization of the compact headpiece conformation.

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