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Characterisation of ATP binding inhibition to the sarcoplasmic reticulum Ca^{2+}-ATPase by thapsigargin

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The inhibition of Ca^{2+}-ATPase of sarcoplasmic reticulum by thapsigargin has been reported to be associated with a suppression of calcium binding to the high affinity transport sites. We report here that thapsigargin also acts as an inhibitor of ATP binding by reducing its apparent affinity by about two orders of magnitude. This inhibition is non-competitive indicating that thapsigargin does not bind to the ATP binding site. This is confirmed by the fact that thapsigargin binding to the Ca^{2+}-ATPase does not affect the binding of 2',3'-*O*-(2,4,6-trinitrocyclohexadienylidene)-ATP (TNP-ATP).

Ca-ATPase; Thapsigargin; ATP

1. INTRODUCTION

Thapsigargin, a sesquiterpene lactone isolated from *Thapsia garganica* L. [1] is known for its capacity to discharge intracellular calcium concentration [2]. This activity is due to the inhibitory effect of thapsigargin on sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [3]. Thapsigargin inhibits the skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase with a dissociation constant lower than 1 nM [4]. The formation of the thapsigargin–enzyme complex is stoichiometric (1:1) and inhibits the Ca^{2+}-dependent hydrolysis activity and the formation of the phosphorylated intermediate [5]. Calcium binding to the Ca^{2+}-ATPase is also affected by thapsigargin [6]: it has also been reported that only a single calcium ion would bind to the Ca^{2+}-ATPase after inhibition by thapsigargin [7] and that this remaining bound Ca^{2+} would not be located on the high-affinity binding external sites, but binds to the enzyme with a relatively lower affinity [8].

From these observations it has been proposed that, in the presence of thapsigargin, the Ca^{2+}-ATPase is stabilized in a dead-end conformation different from the two main E_1 and E_2 states, that are believed to be the extreme stable conformations of the protein [6].

The present study shows how thapsigargin also affects ATP binding on the Ca^{2+}-ATPase reducing its affinity by at least two orders of magnitude in a non-competitive way. We propose a mechanistic interpretation of the inhibition of the Ca^{2+}-ATPase by thapsigargin.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Sarcoplasmic reticulum vesicles were prepared as previously described [9]. Thapsigargin was purchased from LC Services Co. (Woburn, MA) and dissolved in DMSO (stock-solution: 1 mM). TNP-ATP was purchased from Molecular Probes (Eugene, OR).

2.2. *Fluorescence measurements*

Fluorescence signals were measured with a high-sensitivity fluorimeter from Bio-Logic Co. (Claix, France). Experimental conditions were: RS vesicles (100 μg/ml) in 20 mM MOPS-KOH, 100 mM KCl, 200 μM EGTA, 5 mM $MgCl_2$, pH 7.2. The Ca-ATPase intrinsic fluorescence was studied under the following conditions: λ_{exc} = 293 nm; λ_{em} = 336 nm (Corning 320 nm cut-off filter associated with a wide band UV filter). TNP-ATP fluorescence was studied under the following conditions: λ_{exc} = 408 nm, λ_{em} = 560 nm (Balzers K55 broad band filter).

2.3. *Filtration measurements*

Nucleotide binding was measured with a filtration device from Bio-Logic Co., using nitrocellulose filters (Millipore 0.45 μm) at room temperature. SR vesicles (either 10 or 20 mg/ml) were preincubated in 20 mM MOPS-KOH, 100 mM KCl, 5 mM $MgCl_2$, 500 μM EGTA, pH 7.2 and [^{14}C]ATP at various concentrations with or without 20 μM thapsigargin. 50 μl of this preincubated solution were diluted into the binding medium (1 ml) and applied to a filter after various times of incubation. Then the binding solution was passed through the enzyme loaded filter during 30 s. Data were corrected with blanks obtained by omitting the SR vesicles. Filters were dissolved in a scintillation mixture (Beckman) and the radioactivity was measured.

3. RESULTS

3.1. *Measurements of ATP binding to enzyme*

Fig. 1A shows the Mg-ATP concentration depend-

ence of [^{14}C]ATP binding to Ca^{2+}-ATPase and the effect of various thapsigargin concentrations. We observed that the presence of thapsigargin reduced the affinity for ATP of the Ca^{2+}-ATPase in E_2 conformation, changing its dissociation constant from 4 μM to a value that can be estimated as 90 ± 50 μM. The ATP binding was found to be independent to the thapsigargin concentrations as long as the thapsigargin concentration was superior to the concentration of ATPase. Fig. 1B also shows that this shift in affinity is fast and occurs totally within 30 s. No decrease of rate of inhibition was observed even at the highest ATP concentration used. This observation implies that both ATP and thapsigargin are able to bind at the same time to the Ca^{2+}-ATPase in a non-exclusive process.

3.2. *Intrinsic fluorescence measurements*

In the absence of free calcium, Mg-ATP binding to Ca^{2+}-ATPase induces a significant increase of fluorescence, the amplitude of which being about 1/2 of the fluorescence increase due to the transconformation $E_2 \rightarrow E_1$ [10], the apparent affinity for this binding process being 4 μM as for the direct [^{14}C]ATP binding measurements described above. Addition of 2 μM thapsigargin to SR vesicles in the presence of saturating concentrations of Mg-ATP induced a decrease of the fluorescence intensity until a level corresponding to that of the Ca^{2+}-ATPase in the absence of ATP and Ca^{2+} (the E_2 level). This decrease occurred within a few seconds with no apparent effect of the ATP concentration on this rate. Accordingly, when the Ca^{2+}-ATPase was first incubated in excess thapsigargin, addition of Mg-ATP to the SR vesicles did not induce any fluorescence intensity change. Concentrations of ATP up to 1 mM were used, above this concentration the absorbance of ATP concentrations was a too serious limitation to the intrinsic fluorescence measurements.

3.3. *TNP-ATP fluorescence*

TNP-ATP is a fluorescent analogue of ATP whose fluorescence is considerably enhanced upon binding to Ca^{2+}-ATPase [11]. The dissociation constant of TNP-ATP for the Ca^{2+}-ATPase was previously found to be about 400 nM [11]. A clear competition between TNP-ATP and ATP can be observed by measuring the chase of TNP-ATP. Analysis of this competition allows an independent evaluation of the ATP dissociation constant which was found to be in good agreement with the direct ATP binding experiments [11,12].

Surprisingly, addition of thapsigargin to sarcoplasmic reticulum vesicles in the presence of TNP-ATP did not modify the TNP-ATP fluorescence signal intensity nor does it affect its affinity. This result was obtained whatever the incubation time and the thapsigargin concentration. This shows that thapsigargin does not complete at all with TNP-ATP for binding to the nucleotide site. This observation allowed us to perform an independent evaluation of the ATP/thapsigargin competition at high ATP concentrations without the difficulties due to ATP absorbance encountered in the intrinsic fluorescence study. The accuracy of the measurements were, however, limited by the fact that it is a competition between three effectors. The true ATP dissociation constant in the presence of thapsigargin was evaluated by measuring the ATP/TNP-ATP competition for various concentrations of the two ligands.

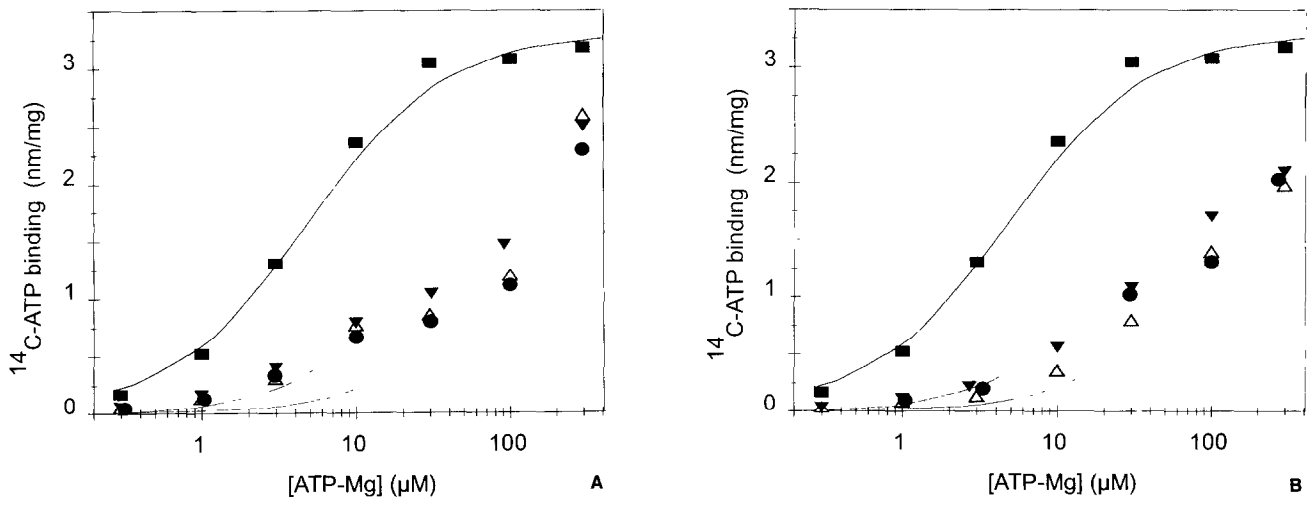

Fig. 1. Effect of thapsigargin on ATP binding. (A) The sarcoplasmic reticulum vesicles were incubated 20 min with various thapsigargin concentrations (■ 0 μM, △ 20 μM, ▼ 40 μM, ● 60 μM) in a medium containing 20 mM MOPS (pH 7.2), 100 mM KCl, 5 mM $MgCl_2$, 500 μM EGTA and various amounts of [^{14}C]ATP. After filtration, the amount of bound ATP is expressed as a function of the total ATP concentration present in the medium. (B) Under similar conditions, the vesicles were incubated with 20 μM thapsigargin for various times (■ 0 min, △ 0.5 min, ▼ 5 min, ● 20 min). The line drawn under the data points are theoretical fits. The line under the uninhibited data has been obtained with a dissociation constant of 4 μM. The area on the right shows the range of variation of acceptable dissociation constant for the inhibited data. The lower and upper limits correspond to 40 and 140 μM, respectively.

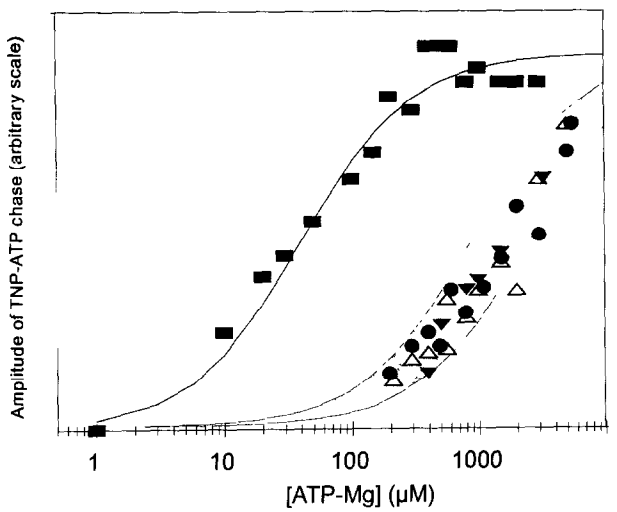

Fig. 2. Thapsigargin effect on the chase of TNP-ATP by ATP. SR vesicles (100 μg/ml) were incubated in the presence of thapsigargin (■ 0 μM, ● 1 μM, △ 2 μM, ▼ 4 μM) in a medium containing 20 mM MOPS (pH 7.2), 100 mM KCl, 5 mM $MgCl_2$, 200 μM EGTA, 2 μM TNP-ATP and the indicated concentration of ATP. The chase of TNP-ATP by ATP is expressed by the decrease of the fluorescence signal of TNP-ATP versus ATP concentration. The line drawn under the data points are theoretical fits. The line under the uninhibited data has been obtained with a dissociation constant of 40 μM. The area on the right shows the range of variation of acceptable dissociation constant for the inhibited data. The lower and upper limits correspond to 0.8 and 2.4 mM, respectively.

Fig. 2 illustrates the effect of thapsigargin on the chase of TNP-ATP by Mg-ATP from its site on the Ca^{2+}-ATPase in the presence of 2 μM TNP-ATP. The presence of thapsigargin shifts the apparent affinity of ATP to the Ca^{2+}-ATPase from 40 μM without thapsigargin, to 1.6 ± 0.8 mM in its presence. From these values, the true affinity for ATP (in the absence of TNP-ATP) was calculated. We obtained 6.7 μM for the free enzyme and 260 ± 130 μM for the enzyme in the presence of thapsigargin.

4. DISCUSSION

Total inhibition of ATP binding by thapsigargin has been reported by Kijima et al. [5]. At variance with this results we show here that the inhibited thapsigargin–enzyme complex is still able to bind ATP with, however, an affinity 20–70 times lower than the native protein.

This reduction of affinity for ATP is uncompetitive as the apparent affinity for ATP of the inhibited enzyme does not depend on the thapsigargin concentration when the inhibitor is added in concentration higher than that of the Ca^{2+}-ATPase. This indicates that the inhibition of ATP binding is not due to a direct competition for binding to the active site nor to any steric hindrance between ATP and thapsigargin, nor to binding to mutually exclusive conformations. This non-competitive effect is confirmed by the fact that TNP-ATP is still able to bind to the thapsigargin-ATPase complex without any observable change of its affinity as compared to that of the native enzyme.

The fact that the competition between TNP-ATP and ATP for binding to the enzyme persists in the presence of thapsigargin has allowed a separate measurement of the dissociation constant of ATP for the thapsigargin–ATPase complex. These measurements confirm the presence of a low affinity ATP binding site on the inhibited enzyme. The dissociation constant found is significantly higher than that measured by direct ATP binding on the thapsigargin–ATPase complex, but the fact that it was measured through a triple competition between thapsigargin, ATP and TNP-ATP limited the accuracy of this series of experiments.

Combining all these evaluations we can conclude that a value of 150 μM is a reasonable estimate for the dissociation constant between ATP and the thapsigargin inhibited Ca^{2+}-ATPase.

Thapsigargin forces the Ca^{2+}-ATPase into a conformation where Ca^{2+} binding is inhibited [6]. It has been also shown that this inhibited state cannot be phosphorylated by P_i [6,7]. The simplest interpretation is that the inhibition of the Ca^{2+}-ATPase by thapsigargin is producing a freezing of the Ca^{2+}-ATPase in the basal E_2 state. This stabilization of the E_2 state, that is evidenced by the level of intrinsic fluorescence, prevents the ATPase to undergo any change of conformation as a result of substrates binding such as that necessary for the phosphorylation by P_i to occur [14,16].

According to this hypothesis the E_2 conformation would then also present a low affinity for ATP (close to 150 μM). In the non-inhibited native ATPase, binding of Mg-ATP to this site induces a conformational change that can be observed by an intrinsic fluorescence increase [10]. The result of this change is the decrease of the observable ATP dissociation contant to a value of 4 μM. This 'induced-fit' process would be inactivated in the thapsigargin–ATPase complex due to immobilization of the E_2 conformation.

REFERENCES

[1] Rasmussen, U., Christensen, S.B. and Sundberg, F. (1978) Acta Pharm. Suec. 15, 133–140.
[2] Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R. and Dawson, A.P. (1990) Proc. Natl. Acad. Sci. USA 87, 2466–2470.
[3] Lytton, J., Westlin, M. and Hanley, M.R. (1991) J. Biol. Chem. 266, 17067–17071.
[4] Sagara, Y. and Inesi, G. (1991) J. Biol. Chem. 266, 13503–13506.
[5] Kijima, Y., Ogunbunmi, E. and Fleischer, S. (1991) J. Biol. Chem. 266, 22912–22918.
[6] Sagara, Y., Wade, J.B. and Inesi, G. (1992) J. Biol. Chem. 267, 1286–1292.
[7] Wictome, M., Henderson, I., Lee, A.G. and East, J.M. (1992) Biochem. J. 283, 525–529.
[8] Wictome, M., Michelangeli, F., Lee, A.G. and East, J.M. (1992) FEBS Lett. 304, 109–113.
[9] Troullier, A., Girardet, J.L. and Dupont, Y. (1992) J. Biol. Chem. 267, 22821–22829.

[10] Dupont, Y., Bennett, N. and Lacapère, J.J. (1982) Ann. NY Acad. Sci. 402, 469.
[11] Dupont, Y., Chapron, Y. and Pougeois, R. (1982) Biochem. Biophys. Res. Commun. 106, 1272–1279.
[12] Watanabe, T. and Inesi, G. (1982) J. Biol. Chem. 257, 11510–11516.
[13] Inesi, G. and Sagara, Y. (1992) Arch. Biochem. Biophys. 298, 313–317.
[14] Dupont, Y. and Pougeois, R. (1983) FEBS Lett. 156, 93–98.
[15] Susuki, H., Kubota, T., Kubo, K. and Kanazawa, T. (1990) Biochemistry 29, 7040–7045
[16] Lacapere, J.J., Gingold, M.P., Champeil, P. and Guillain, F. (1981) J. Biol. Chem. 256, 2302–2306.