Derivatives of thapsigargin as probes of its binding site on endoplasmic reticulum Ca^{2+} ATPase

Stereoselectivity and important functional groups

Søren Brøgger Christensen[a], Annette Andersen[a], Jens-Christian J. Poulsen[b], Marek Treiman[b,*]

[a]PharmaBiotec, Department of Organic Chemistry, The Royal Danish School of Pharmacy, University of Copenhagen, Copenhagen, Denmark
[b]Department of Medical Physiology, Biotechnology Center for Signal Peptide Research, University of Copenhagen, Panum Institute, Blegdamsvej 3C, DK-2200 N Copenhagen, Denmark

The naturally occurring sesquiterpene lactone thapsigargin is a potent and selective inhibitor of SERCA ATPases, a family of Ca^{2+}-pumping ATPases present in the endoplasmic reticulum of all mammalian cells. We have studied some of the molecular features of thapsigargin responsible for its inhibitory action towards these Ca^{2+} ATPases. A series of thapsigargin analogues were synthesised and their inhibitory potencies determined using the uptake of $^{45}Ca^{2+}$ in bovine cerebellar microsomes as a sensitive marker of Ca^{2+} ATPase activity. An attenuation of the inhibitory potency relative to the parent compound was found ranging from slight to over 3 orders of magnitude. The inhibitory activity showed a very strong configuration dependence, a major contribution from the ester groups at C3 and C10, and an apparently minor contribution from the lactone ring substituents. The data are consistent with thapsigargin fitting to a sterically discriminating cleft involving the hydrophobic transmembrane region of the ATPase, and is compatible with available kinetic evidence of thapsigargin-mediated inhibition.

Thapsigargin; Ca^{2+} ATPase; Endoplasmic reticulum; Ca^{2+} store

1. INTRODUCTION

Correct distribution of Ca^{2+} ions within the cellular compartments is required for their well-established function as molecular signals in eukaryotic cells [1,2]. Endoplasmic reticulum (ER) (or its specialized domains) plays an important role in this compartmentalization [2,3]. ATP-dependent Ca^{2+} uptake from the cytosol to ER lumen is a prerequisite for rapid cytosolic signaling through receptor-mediated Ca^{2+} release [4], and for the role of ER-luminal Ca^{2+} in protein synthesis, maturation and sorting [5,6].

This ATP-requiring Ca^{2+} transport to the ER lumen is accomplished by a family of ER Ca^{2+} ATPases, termed SERCA ATPases [7]. Despite the wealth of information accumulated about the molecular structure and function of these ion pumps (for recent reviews see [8,9]), important features of their operation require clarification. These include the precise location of catalytic Ca^{2+} binding sites, as well as the nature of the molecular rearrangements involved in the translocation of Ca^{2+} [10]. Thapsigargin, a sesquiterpene lactone [11] shown to selectively inhibit all of the SERCA isozymes with subnanomolar potency [12] has in recent years provided new ways of studying the molecular function of ER Ca^{2+} ATPases. Upon binding, thapsigargin appears to induce a conformational state of the pump in which several of the partial reactions (e.g. Ca^{2+} binding, Ca^{2+}-independent phosphorylation by P_i, nucleotide binding), spanning rather distant domains of the ATPase, are blocked [13]. Thus, understanding of the molecular basis of the thapsigargin-ATPase interaction, including the location of the thapsigargin-binding site on the enzyme, would contribute significantly to the major goal of elucidating its molecular operation. With this goal in mind, we have synthesized a number of thapsigargin analogues and tested their ability to act as inhibitors of ER Ca^{2+} ATPase.

2. MATERIALS AND METHODS

2.1. Preparation of microsomes

Bovine cerebella were obtained from a local abbatoir, transported on ice and processed within 2–3 h of the animal's death. All the subsequent preparation steps were also carried out on ice. Homogenization was carried out in a buffer (HB) containing; 20 mM TES, pH 7.0; 300 mM sucrose; 0.1 mM phenylmethylsulfonyl fluoride, by means of a loosely fitting pestle in a glass-teflon homogenizer, 6 strokes at 1000 rpm. The material was then spun at $600 \times g_{av}$ for 10 min. The supernatant was spun again at $10,000 \times g_{av}$ for 10 min, and the resulting supernatant was centrifuged at $106,000 \times g_{av}$ for 1 h. The pellet, resuspended in HB, constituted the microsomal fraction. It was

*Corresponding author. Fax: (45) 35 32 75 37.

aliquoted and stored for several weeks at −80°C without loss of the ATP-dependent $^{45}Ca^{2+}$ uptake activity.

2.2. Synthesis of thapsigargin analogues

These compounds were synthesised as described in [14].

2.3. Measurement of the ATP-dependent $^{45}Ca^{2+}$ uptake

This was carried out at 37°C in 1 ml of a medium containing (concentrations in mM): TES, 20 (pH 7.0); KCl, 110; $MgCl_2$, 5; potasssium oxalate, 6; EGTA, 0.5; $^{45}CaCl_2$ (about 15,000 cpm/nmol), 0.21 (resulting in 0.3 μM free Ca^{2+}), ATP 2; microsomal protein 50 μg. Thapsigargin or its analogues in dimethylsulfoxide (DMSO) (or a corresponding amount of DMSO alone, final concentration 0.5% v/v) were added 12 min before starting the uptake by adding ATP in TES buffer (or an equivalent volume of TES for blank determinations). The incubations were stopped (after 10 min in routine experiments) by filtering the samples through Whatman GF/F filters (presoaked in polyethylene imine 0.1%) on a Brandell Harvester instrument. The filters were immediately washed using an ice-cold solution of Tris 20 mM (pH 7.0); $MgCl_2$ 10 mM; NaCl 140 mM, and collected for scintillation counting at 100% efficiency.

2.4. Calculations

The ATP-dependent component of $^{45}Ca^{2+}$ uptake in the absence of any inhibitors was normalized to 100%. The dose-dependent curves and inhibitor IC_{50} values were obtained by fitting 4-parameters logistic equation to the data by means of the program GRAFIT (Erithacus Software Ltd.). Free Ca^{2+} concentrations in the presence of EGTA, ATP and Mg^{2+} were calculated by means of a computer brogram kindly provided by Prof. M. Gratzl, Ulm University.

2.5. Other methods

Protein was determined by the fluorescamine method [15].

3. RESULTS

The insert in Fig. 1 shows that in the presence of oxalate, the time–course of the $^{45}Ca^{2+}$ uptake due to the ER Ca^{2+} ATPase in cerebellar microsomes was linear for at least 30 min at low (0.3 μM) as well as high (10 μM) concentrations of free Ca^{2+}. (At 0.3 μM free Ca^{2+}, the linearity was preserved between 20 and 100 μg of

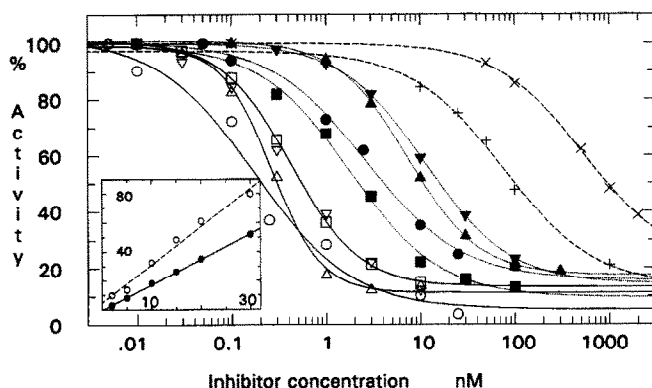Fig. 1. Dependence of the $^{45}Ca^{2+}$ uptake inhibition in bovine cerebellar microsomes on the concentrations of various thapsigargin derivatives. Symbols indicate the individual drugs as follows: ○, 1; △, 2; □, 3; ▽, 4; ■, 5; ●, 6; ▲, 7; ▼, 8; +, 9; ×, 10. (See Fig. 2 for explanations of the drug numbers. Points are means of n independent determinations, with n values indicated in Fig. 2.) (Insert) Linear time–course of $^{45}Ca^{2+}$ uptake under the conditions used for testing the inhibition due to thapsigargin derivatives. Abscissa: time in min. Ordinate: nmol $^{45}Ca^{2+}$/ mg protein. Free Ca^{2+} (μM): ○, 10; ●,0.3.

microsome protein/assay tube; data not shown.) Fig. 2 shows the inhibitors employed in this work, together with their respective IC_{50} values derived from the dose–response curves of Fig. 1. For each of the inhibitors, the factor indicating its potency decrease relative to thapsigargin is also given as the R_{IC} value. The inhibitory potencies of the thapsigargin derivatives were all smaller than that of the parent substance. This attenuation of the inhibitory potencies spanned a wide range, from barely significant to 3 orders of magnitude. Based on the R_{IC} values in Fig. 2, the compounds may be divided into three groups. Group I consists of compounds 1–4. Group II consists of compounds 5–8. Group III contains compounds 9 and 10. In group I, compounds 3 and 4 were obtained by acetylation of one of the OH groups on the lactone ring (at C11 and C7, respectively). These changes resulted in small (less than 3-fold) shifts of the inhibitory potency relative to thapsigargin. Isomerisation of the double bond of the angelic acid residue in 1 to give 2 did not afford any appreciable shift of the IC_{50} value.

Compounds of group II showed moderate-to-strong shifts in the inhibitory potency. The strongest potency shifts in this group were observed with compounds 7 (hydrolysis of acetate at C10) and 8 (conversion of the angelic ester to a ketone at C3). In contrast, compounds 5 and 6 represent an introduction of new groups: in 5, the angeloate at C3 was replaced by an octanoate, while in compound 6, both of the OH groups were acetylated. Introduction of these groups to obtain compounds 5 and 6 resulted in a 3- to 6-fold smaller attenuation of ATPase inhibition than removing the ester groups at C10 or C3 (to obtain compounds 7 and 8).

Group III consists of compounds 9 and 10, showing the largest potency shifts (over 400- and 3,000-fold, respectively) relative to thapsigargin. Compound 10 is a diastereoisomer of thapsigargin, in which the configuration at C8 has been inverted. Compound 9 is a diastereoisomer of 5, in which the configuration at C3 has been inverted. These inversions resulted in a trans disposal of the substituents at C3 and C8 in each of the compounds 9 and 10.

4. DISCUSSION

4.1. ATP-dependent $^{45}Ca^{2+}$ uptake as an expression of the ER-type (SERCA) Ca^{2+}-ATPase activity in bovine cerebellar microsomes

In the cerebellum, the SERCA 2b form of ER Ca^{2+} ATPase appears strongly predominant [16,17], with the level of expression among the highest in the brain [17,18]. The high sensitivity of $^{45}Ca^{2+}$ uptake assay, as used in the present work, was primarily due to two factors. Firstly, the non-specific (i.e. ATP-independent) $^{45}Ca^{2+}$ binding to the filters was only 5–10% of the total uptake. Secondly, addition of oxalate ion to the $^{45}Ca^{2+}$ uptake medium allowed this uptake to proceed in a
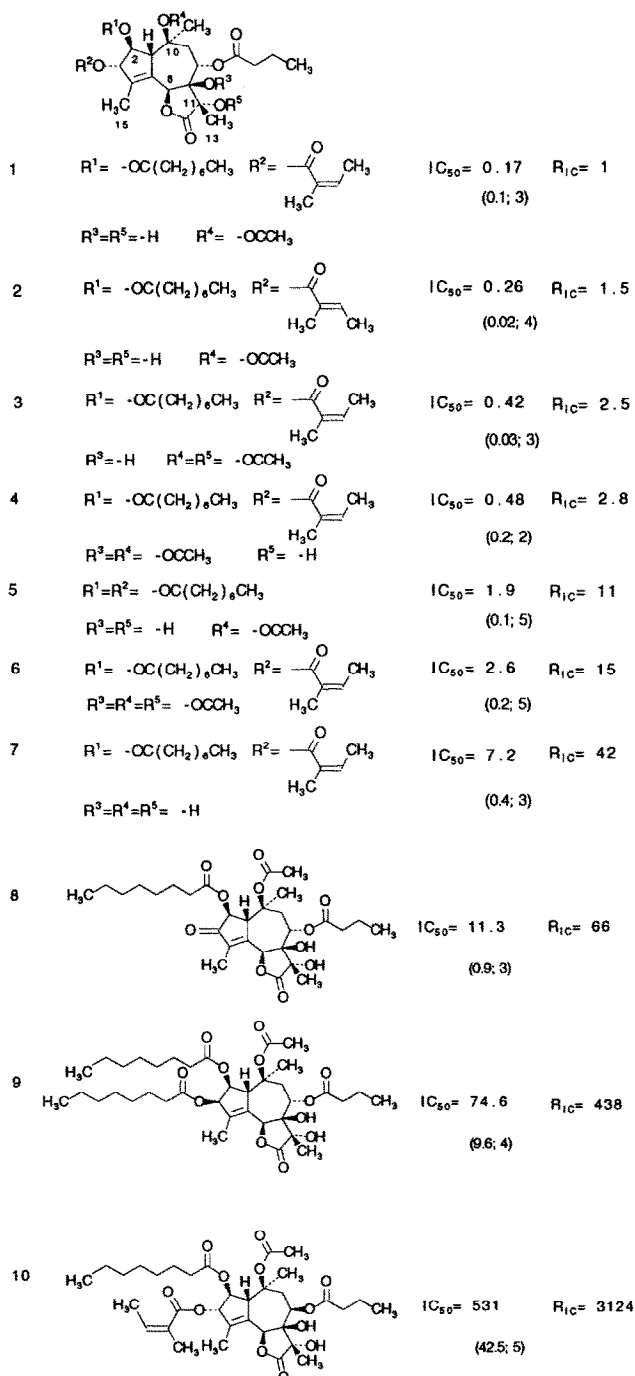
Fig. 2. Thapsigargin derivatives and their inhibitory potencies. Drugs 1–10 are defined as shown. IC_{50} values (nM) are means of n determinations, each based on fitting an independent set of points, with SEM (or range for $n = 2$) and n values indicated by numbers in the brackets. R_{IC} values were obtained by dividing the corresponding IC_{50} values by the IC_{50} value of unmodified thapsigargin (drug 1).

linear fashion for at least 30 min (Fig. 1, insert), due to the precipitation of the ^{45}Ca–oxalate complex in the lumen of ER vesicles stabilizing the intravesicular free ^{45}Ca^{2+} at a constant value [19]. This enhancement of the uptake by oxalate occurs mainly in the ER-derived vesicles [20], effectively diminishing the contribution of

non-ER-type Ca^{2+} pumps (e.g. in plasma membrane vesicles oriented inside-out, or internal mitochondrial membranes) to the measured ^{45}Ca^{2+} uptake. Fig. 1 shows that the contribution of such non-ER-type Ca^{2+}-ATPases was about 15%, as indicated by that portion of uptake which could not be inhibited by thapsigargin or its analogues.

4.2. Consequences of structural modifications of thapsigargin for the inhibitory potency

The molecular features of thapsigargin of potential importance for its interaction with Ca^{2+}-ATPases include the lipophilicity, the spatial configuration, and the presence of substituents which provide sites of specific interactions with the protein. The lipophilic nature of thapsigargin implies that those portions of the enzyme located within the lipid bilayer might a priori be considered a prime target for the drug. This possibility is indeed supported by the kinetic evidence. This evidence suggests that thapsigargin does not bind within the ATP-binding domain, located in the bulky, extra-membraneous portion of ATPase [13,21]. On the other hand, thapsigargin was found to stabilize the transmembrane region of the ATPase [22]. These observations, together with the binding stoichiometry of 1 mol thapsigargin/ mol ATPase, are consistent with the possibility that a domain including amino acids from one or more of the ATPase transmembrane α-helices is responsible for thapsigargin binding. The data to be discussed below would be consistent with such an intra-membraneously located domain being sterically constrained to form a 'cleft', discriminating between thapsigargin analogues of different configurations.

Until more definite information becomes available about the composition of this thapsigargin binding domain, interpretation of our present data in terms of specific drug–protein interactions must remain somewhat tentative. With this caveat in mind, in the following we point out some structural features of thapsigargin which our data suggest to be of importance for its inhibitory activity, and which should aid attempts to define the excluded and essential volume [23] of the enzyme as a step towards the mapping of the binding site.

The compounds of group I (2–4) showed only minor decreases of the inhibitory potency relative to the parent drug (1). The modest effects of acetylation of the OH groups at the lactone ring in 3 and 4 would suggest that this part of the molecule is only loosely involved in the binding to the ATPase. (This is further supported by a potent inhibition exerted by an analogue in which the carbonyl group at C12 has been reduced S.B. Christensen and M. Treiman, unpublished). However, acetylation of both hydroxy groups (compund 6, see below) affords a stronger reduction of the pump inhibitory activity than acetylation of any of these groups alone. This would suggest either that the presence of two ace-

tate groups makes this part of the molecule too volumi-
nous, impeding its access to the binding cleft, or that the
presence of a hydrogen donor at the lactone ring substit-
uent(s) is of importance.

Group II (5–8) consists of two types of compounds:
those substituted with voluminous groups (5 and 6), and
those in which acyl groups have been removed (7 and
8). Replacement of the angeloyl group of thapsigargin
with the voluminous octanoyl group in 5 results in a
10-fold loss of the inhibitory potency, consistent with
the idea that the angeloyl group binds within a sterically
limited cleft. The aliphatic side chain extension might
incur a steric disadvantage not compensated for by an
increased potential for hydrophobic interactions. The
considerable loss of inhibitory potency of 8 in which the
angeloate group has been removed and replaced with a
ketone group confirms that this part of thapsigargin
might be intimately associated with the ATPase. Possi-
ble interactions might include hydrogen bonding to the
ester carbonyl group, interactions of the carbonyl group
electrons with amino acid aromatic rings or hydropho-
bic interactions. By analogy, the decreased inhibitory
potency of 7 suggests that the acetate residue at C10 is
of importance for the binding to the enzyme.

The present data are not sufficient to separate une-
quivocally the effects on the inhibitory potency due to
the gross steric changes (for instance through a shape
of the drug highly disadvantageous for the access to the
binding cleft) from more subtle effects on specific inter-
actions (for instance through weakening interactions
between specific group(s) on the drug and the binding
domain of the protein through misalignment). Thus
both kinds of effects might contribute to the very pro-
nounced potency shifts observed with the compounds
in group III (9 and 10). Nevertheless, the severe reduc-
tion in inhibitory potency by epimerisation clearly dem-
onstrates the stereo-specificity of the interaction be-
tween thapsigargin and its binding domain on the ATP-
ase.

In conclusion, by studying a number of thapsigargin
analogues, we have demonstrated that selective modifi-
cations of this drug result in a stepwise decrease of
inhibitory potency over a wide range. The high inhib-
itory potency of the thapsigargin is shown to be criti-
cally dependent on its configuration, and is consistent
with the idea of the drug fitting to a cleft involving a
hydrophobic region of the ATPase. A definite assign-
ment of the contributions to the binding from the indi-
vidual groups of the thapsigargin molecule is not possi-
ble at present. However, a suggestion may be made of
the relative importance of carboxylic acid substituents

at C3 and C10, and a comparatively minor contribution
from the lactone ring. These observations provide a step
towards mapping of the thapsigargin-binding domain
on ER-type Ca^{2+}-ATPases, in an effort to elucidate the
molecular operation of these pumps.

REFERENCES

[1] Cheek, T.R. (1991) Curr. Opin. Cell Biol. 3, 199–205.
[2] Pietrobon, D., Di Virgilio, F. and Pozzan, T. (1990) Eur. J.
    Biochem. 193, 599–622.
[3] Meldolesi, J., Madeddu, L. and Pozzan, T. (1990) Biochem. Bio-
    phys. Acta 1055, 130–140.
[4] Berridge, M.J. (1993) Nature 361, 315–325.
[5] Brostrom, C.O. and Brostrom, M.A. (1990) Annu. Rev. Physiol.
    52, 577–590.
[6] Gething, M.-J. and Sambrook, J. (1992) Nature 355, 33–45.
[7] Burk, S.E., Lytton, J., MacLennan, D.H. and Shull, G.E. (1989)
    J. Biol. Chem. 264, 18561–18568.
[8] Inesi, G., Sumbilla, C. and Kirtley, M.E. (1990) Physiol. Rev. 70,
    749–760.
[9] MacLennan, D.H., Clarke, D.M., Loo, T.W. and Skerjanc, I.S.
    (1992) Acta Physiol. Scand. 146, 141–150.
[10] Andersen, J.P. and Vilsen, B. (1992) Acta Physiol. Scand. 146,
    151–159.
[11] Christensen, S.B., Andersen, A., Lauridsen, A., Moldt, P., Smitt,
    U.W. and Thastrup, O. (1992) in: New Leads and Targets in Drug
    Design (Krogsgaard-Larsen, P., Christensen, S.B. and Kofod, H.
    eds) pp. 243–252, Munksgaard, Copenhagen.
[12] Lytton, J., Westlin, M. and Hanley, M.R. (1991) J. Biol. Chem.
    266, 17067–17071.
[13] Inesi, G. and Sagara, Y. (1992) Arch. Biochem. Biophys. 298,
    313–317.
[14] Andersen, A., Cornett, C., Lauridsen, A., Olsen, C.E. and Chris-
    tensen, S.B., Acta Chem. Scand., submitted.
[15] Bohlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973)
    Arch. Biochem. Biophys. 155, 213–220.
[16] Plessers, L., Eggermont, J.A., Wuytack, F. and Casteels, R.
    (1991) J. Neurosci. 11, 650–656.
[17] Miller, K.K., Verma, A., Snyder, S.H. and Ross, C.A. (1991)
    Neuroscience 43, 1–9.
[18] Michelangeli, F., Divirgilio, F., Villa, A., Podini, P., Meldolesi,
    J. and Pozzan, T. (1991) Biochem. J. 275, 555–561.
[19] Hasselbach, W. (1964) Prog. Biophys. Mol. Biol. 14, 169–222.
[20] Mathiasen, D., Røssum, L.M., Robinson, I.M., Burgoyne, R.D.,
    East, J.M., Møller, M., Rasmussen, H.N. and Treiman, M.
    (1993) Int. J. Biochem. 25, 641–652.
[21] Sagara, Y., Fernandezbelda, F., Demels, L. and Inesi, G. (1992)
    J. Biol. Chem. 267, 12606–12613.
[22] De Meis, L. and Inesi, G. (1992) FEBS Lett. 299, 33–35.
[23] Hacksell, U. (1991) in: A Textbook of Drug Design and Develop-
    ment (Krogsgaard-Larsen, P. and Bundgaard, H. eds.) pp. 26–54,
    Harwood, Chur.