

A study of calcium release from rat liver microsomes by thapsigargin induction *

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Summary: The primary aim of the study was to test the influences of thapsigargin in releasing Ca^{2+} from the intracellular Ca^{2+} stores and comparing it with the effects of thapsigargin. In this study, the effect of tumour promoter thapsigargin on intracellular Ca^{2+} has been described. Therefore, rat liver endoplasmic reticulum subcellular fractions (microsomes) were employed and Ca^{2+} movements measured by spectrofluorimeter. Fluo-3 studies were used to follow Ca^{2+} release in experiments with microsomes. When results are evaluated, it has been concluded that like thapsigargin, thapsigargin has the ability of discharging the intracellular Ca^{2+} stores, increasing the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and being a potent and specific inhibitor of the ER Ca^{2+} -ATPase.

Key words: Ca^{2+} release, intracellular stores, SERCA ATPases, spectroscopy, thapsigargin (TG), thapsigargin (TGC).

Tapsigarginin indüksiyonu ile sıçan karaciğer mikrozoamlarından kalsiyum salınımı üzerine bir çalışma

Özet: Bu çalışmanın ana amacı hücre içindeki Ca^{2+} depolarından Ca^{2+} salınımına tapsigarginin etkisinin test edilmesi ve bunun tapsigargin etkisi ile karşılaştırılmasıdır. Çalışmada tümör promotörü tapsigarginin hücre içindeki Ca^{2+} a etkileri tanımlanmıştır. Bu nedenle, sıçan endoplazmik retikulum subsellüler (hücrealtı) fraksiyonları (mikrozoamlar) kullanılmış ve Ca^{2+} hareketleri spektrofotometre ile ölçülmüştür. Mikrozoamlardaki Ca^{2+} salınımı Fluo-3 çalışmaları ile takip edilmiştir. Elde edilen bulgular değerlendirildiğinde, tapsigarginin de tapsigargin gibi hücre içi Ca^{2+} depolarını boşalttığı, hücre içi serbest Ca^{2+} konsantrasyonunu ($[Ca^{2+}]_i$) arttırdığı ve ER Ca^{2+} -ATPaz için güçlü ve özgül bir inhibitör olduğu kanısına varılmıştır.

Anahtar sözcükler: Ca^{2+} salınımı, hücre içi depolar, SERCA ATPaz, spektroskopisi, tapsigargin (TG), tapsigargin (TGC).

Introduction

The survival, specialized functions and reproduction of eukaryotic cells and many prokaryotes requires Ca^{2+} in the medium bathing the cells. Intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) and the concentration of Ca^{2+} in the intracellular stores [(endoplasmic reticulum (ER) and its muscle counterpart, sarcoplasmic reticulum (SR)] are controlled by the network of Ca^{2+} channels and transporters (pumps). Ca^{2+} -ATPase pumps are one of those recognised mechanisms that are found in the plasma membrane (Plasma membrane Ca^{2+} -ATPase: PMCA), in the ER/SR membrane (sarco-endoplasmic Ca^{2+} -ATPase: SERCA), in the Golgi and in the nuclear envelope (in yeasts, they are also found in other organelles). They export Ca^{2+} to the ER/SR lumen or to the extracellular spaces (2).

The SERCA ATPase transports Ca^{2+} ions into the SR with a stoichiometry of $2Ca^{2+}: 1ATP$, which suggests a major mechanistic difference to the PMCA, as the latter

works with a $1Ca^{2+}:1$ ATP stoichiometry (21). The understanding of the molecular mechanism of the SERCA pump has been greatly advanced by the recent solution of its crystal structure at 2.6 Å resolution (29) and the published structure indeed has two Ca^{2+} ions bound to the transmembrane domain (3). Although both pumps (PMCA and SERCA) have ten putative transmembrane-spanning domains, homology between the two classes is unexpectedly low (18).

On the other hand, according to the findings of Dawson (8), it thus appears that the SR and ER Ca^{2+} -ATPases are probably not identical, but there are certainly great similarities between them. In the following years, the ER Ca^{2+} transport ATPase of blood platelets were compared with the Ca^{2+} -ATPase of SR skeletal muscle. The stoichiometry between Ca^{2+} ion released and ATP synthesized by platelet Ca^{2+} -ATPase was found to be 1, while that of skeletal muscle was 2. The possibility was discussed that the differences

* Summarized from the MSc dissertation of the author. The study was carried out in accordance with the ethical rules and recommendations.

observed between the two transport systems is related to the distinct amino acid sequences of the enzymes (1).

There are a number of different chemical compounds which inhibit specifically the SERCA ATPases, like thapsigargin (TG), thapsigarginin (TGC), cyclopiazonic acid (CPA), and 2,5-di(tert-butyl)hydroquinone (DBHQ) (Figure 1). From those, CPA is one of the toxic metabolites produced by the molds *Penicillium cyclopium* and *Aspergillus flavus* (13). Goeger et al. (12) found that CPA is a potent inhibitor of Ca^{2+} sequestration and ATPase activity in SR of rat skeletal muscle and suggested that this inhibition accounts for CPA toxicity. Hydroquinones are important industrial chemicals (20) and DBHQ was found to inhibit ATP-dependent Ca^{2+} uptake by liver microsomes. However, it was ineffective on Ca^{2+} transport by mitochondria or plasma membrane (19), so its mechanism quite similar to that of TG (30) (see below).

Today, the known range of collectively termed ‘thapsigargin family’ found in *Thapsia* genus had reached 16 members (including TG and TGC) and these natural compounds fall into two series of molecules differentiated by the presence of an oxygen substituent at the C2 position (15). TG, is a tumor-promoting sesquiterpene lactone, isolated from the root of the umbelliferous plant *Thapsia garganica* L. (5, 24) and is commonly used as a probe for intracellular Ca^{2+} storage and release processes. From previous studies, the general characteristics of cellular TG’s on Ca^{2+} responses can be summarized as follows; (i) a $[\text{Ca}^{2+}]_i$ rise that is slower than that elicited by hormonal stimulation, (ii) no concomitant breakdown of inositolphospholipids with the production of inositol phosphates (14, 26), and (iii) an

initiation of $[\text{Ca}^{2+}]_i$ rise by release of intracellular sequestered Ca^{2+} .

According to Christensen et al. (6), the activity of TG originates mainly from the ester groups at C3, C8 and C10 positions and also from the lactose ring substituents. Comparison of the structures of TG and TGC (MWs are; 652,79 and 622,70 respectively) reveals that the only difference between the two compounds is the presence of four methylene groups in the acyl group of TGC, whereas six are present in TG (Figure 1). By considering their close structure to each other and their origin of same umbelliferous plant, the main aim of this study was to show the effects of TGC, as SERCA ATPase inhibitor, on Ca^{2+} movements by using rat liver microsomes and comparing its effects with other well known SERCA ATPase inhibitor TG.

Materials and Methods

Animals, chemicals, equipments: Healthy, male Wistar albino rats weighing 200-250 g. were used in this study. Fluo-3 pentammonium salt (a membrane impermeable Ca^{2+} indicator) was purchased from Molecular Probes Inc. (Eugene, OR, U.S.A). Phosphocreatine, creatine phosphokinase, A23187 (a calcium ionophore), EGTA (ethylene glycol-bis(beta-aminoethylether)-N,N,N',N'-tetraacetate) and BSA (bovine serum albumin) were purchased from Sigma Chemical Co., Poole, Dorset, U.K. ATP and DTT (dithiothreitol) were from B.C.L. (Lewes, Sussex, UK). Thapsigarginin (TGC) was purchased from LC Laboratories (Nottingham, UK). All other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

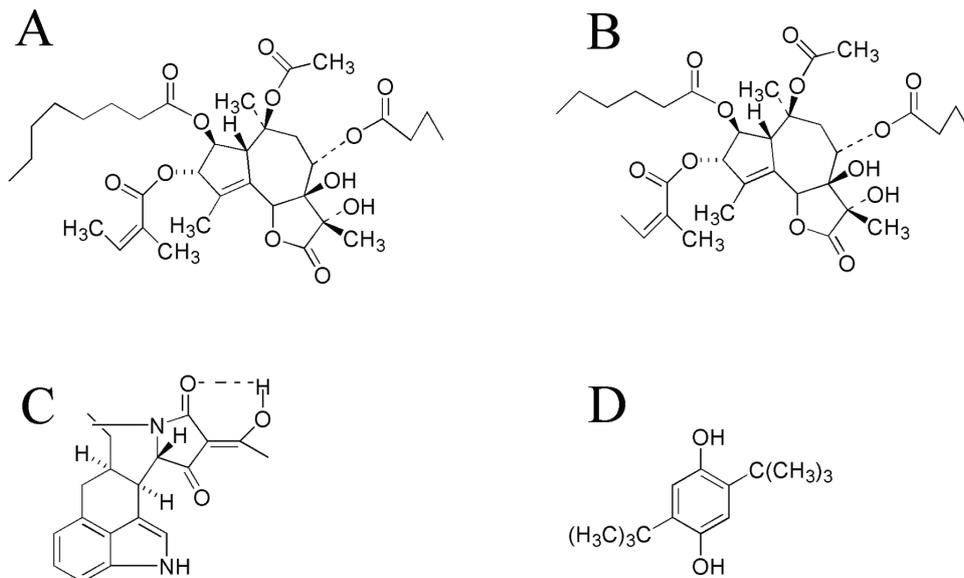


Figure 1. Structures of Ca^{2+} -ATPase inhibitors

A. Thapsigargin (TG), B. Thapsigarginin (TGC), C. Cyclopiazonic acid (CPA), D. 2,5-Di(tert-butyl)hydroquinone (DBHQ)

Şekil 1. Ca^{2+} -ATPaz inhibitörlerinin yapıları

A. Tapsigargin (TG), B. Tapsigarginin (TGC), C. Siklopiazonik asit (CPA), D. 2,5-Di(tert-butil)hidrokinon (DBHQ)

Spectrophotometric studies employed a Shimadzu RF-5000 fluorimeter. The fluorimeter was used under mode of operation of single wavelength spectrofluorimetry for Ca^{2+} release experiments by using Fluo-3 pentammonium salt. The commercially available Shimadzu RF-5000 fluorimeter has a minimum mixing time of 1 s.

Preparation of rat liver endoplasmic reticulum vesicles (microsomes): Microsomes were prepared from the livers of fed 200-250 g. male Wistar albino rats according to Dawson (8) and Reinhart and Bygrave (9) with some modifications. The liver was dissected and immediately suspended in 100 ml of ice-cold homogenization buffer (250 mM sucrose, 5 mM Hepes/KOH, pH 7.0, 1 mM EGTA and 1 mM DTT). All relevant steps were carried out at 4°C. The liver was chopped and homogenized by six strokes of a Dounce homogenizer (0.2 mm radial clearance). This homogenate was centrifuged at 3000 g for 5 min. in a Sorvall RC-5B centrifuge and the supernatant was recentrifuged at 8000 g for 10 min. to give a pellet termed the mitochondrial fraction. Microsomes were then prepared from the resulting supernatant by centrifugation at 18000 g for 20 min. The pellet was resuspended in a second buffer (250 mM sucrose, 5 mM Hepes/KOH, pH 7.0, 10 mM KCl and 1mM DTT) and centrifuged (18000 g, 20 min). This final spin produced a microsomal pellet which was resuspended in a small volume of the same (second) buffer and homogenized in a small volume homogenizer (1 ml) to give a protein concentration of 35-45 mg/ml as determined by the method of Lowry (16). Bovine serum albumin (BSA; Sigma fraction V) was used as a standard.

Ca^{2+} uptake movement in rat liver endoplasmic reticulum vesicles (microsomes): The assay mixture, routinely used for rat liver microsomes, contained in a final volume of 1 ml: 150 mM sucrose, 50 mM KCl, 10 mM Hepes/KOH, pH 7.0, 5 % (w/v) polyethylene glycol, 1 mM DTT, 2 mM MgCl_2 , 5 mM ATP and an ATP regenerating system consisting of 50 $\mu\text{g/ml}$ creatine phosphokinase and 5 mM phosphocreatine. An ATP regenerating system allows a greater degree of Ca^{2+} uptake. The temperature was 30°C and uptake was started by the addition of 1.2 mg/ml microsomal protein. The movements of Ca^{2+} were determined by using Fluo-3 dye. 1 μM Fluo-3 (pentammonium salt) was added to the assay medium before starting Ca^{2+} uptake. All fluorescence measurements were made using a Shimadzu RF-5000 fluorimeter. A magnetic stirring device ensured quick and complete mixing of any additions. To show the effects of TGC for rapid and complete Ca^{2+} release from rat liver microsomes and its dose-dependent effect, both single (64nM) and complementary amounts (1.6 nM, 8 nM, 16 nM, 38.4 nM) of TGC were added into the

separate sets of assay mixtures. With the omission of ATP regenerating system, the dose-dependent effect of TGC were also tested for the microsomal protein concentration by using microsomes at both low (0.56 mg/ml) and high (1.12 mg/ml) concentrations, with the range of 0-16 nM TGC and 0-64 nM TGC, respectively. The initial rate of Ca^{2+} uptake was expressed as nmol Ca^{2+} /mg/min. The number of experiments (or liver used) (n) = 7.

Determination of ATPase activity by using rat liver endoplasmic reticulum vesicles (microsomes): The ATPase activity of the microsomal fraction was determined by measurement of P_i release from ATP according to Dawson and Fulton (10). The incubation mixture contained in a final volume of 1 ml: 100 mM KCl, 20 mM Hepes/KOH, pH 7.0, 5 mM MgCl_2 , 1 $\mu\text{g/ml}$ A23187, 50 μM CaCl_2 (to give the required free Ca^{2+} concentration, approximately 5 μM) and 0.7 - 2.0 mg of microsomal protein. This medium describes the 'plus Ca^{2+} conditions', whereas the 'minus Ca^{2+} conditions' only differ in so far as added Ca^{2+} was excluded and replaced by 1 mM EGTA (free Ca^{2+} < 1nM). For both plus and minus Ca^{2+} conditions, the Ca^{2+} -ATPase activity of rat liver microsomes were detected for a range of 0-320 nM TGC concentrations, with number of experiments (or liver used) (n) = 7 and 4, respectively. After pre-incubation at 37°C for 5 min., the reaction was started by the addition of 1 mM ATP. After incubation (37°C, 10 min.), the reaction was terminated by the addition of 0.5 ml of 5% (w/v) cold trichloroacetic acid (TCA). The resultant precipitate was centrifuged using a bench centrifuge (Sigma 3K10-Howe) and 1 ml of the supernatant was removed. The inorganic phosphate was measured by a method described by Fiske and Subbarow (11). Blanks were also used to set up the spectrophotometer and samples were read at 660 nm.

Results

Thapsigargin-induced Ca^{2+} release from rat liver microsomes: Addition of thapsigargin (TGC^a ; 64 nM) to Ca^{2+} loaded rat liver microsomes results in the rapid and complete release of the sequestered Ca^{2+} (Figure 2A). This result is consistent with the thapsigargin-induced Ca^{2+} release data described by Thastrup et al. (27). The effects of complementary amounts of TGC ($\text{TGC}^{b,c,d,e}$) can be seen in Fig. 2B. According to this figure it is possible to suggest that, like thapsigargin, TGC has a dose-dependent kind of effect on Ca^{2+} release from microsomes. As it can also be seen from the same figure, after releasing all sequestered Ca^{2+} from the TGC-releasable pool, additional amounts of TGC, even maximum concentrations (TGC^a), do not cause any further Ca^{2+} release from the vesicles even though a

small further release is given by A23187. Therefore it is possible to suggest that for rat liver endoplasmic reticulum microsomes, thapsigargin appears to cause irreversible Ca^{2+} release, maximal release is given by 64 nM. The comparison of the data from Figure 2B together with the results shown in Figure 3, reinforce the idea of the dose-dependence of TGC on Ca^{2+} release from microsomes. Figure 3 also shows that the concentration of TGC required to cause Ca^{2+} uptake depends on the microsomal protein concentration. At low protein concentrations (0.56 mg/ml) the dose-response curve shifts to the left. This suggests that, like TG, TGC is a very high affinity inhibitor which binds stoichiometrically to its target.

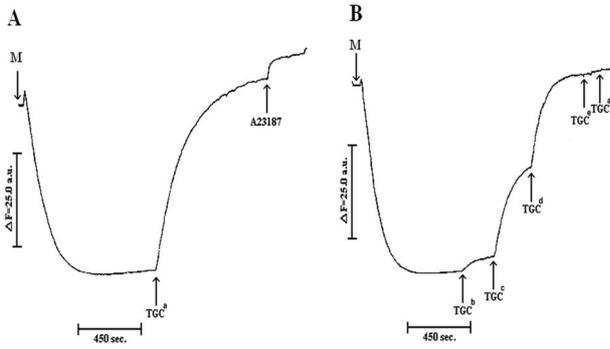


Figure 2. Thapsigargin-induced Ca^{2+} movements in rat liver microsomes. In **A**, 64 nM thapsigargin (TGC^a) was added at 6 min. after initiating Ca^{2+} uptake upon addition of 1 mg/ml microsomes (M), as indicated by the arrows. Complete store depletion was facilitated by addition of A23187 (1 $\mu\text{g}/\text{ml}$) at the time indicated by the arrow. **B**, the same as **A**, however complementary amounts of thapsigargin (TGC^b ; 1.6 nM, TGC^c ; 8 nM, TGC^d ; 16 nM, TGC^e ; 38.4 nM) was added after initiating Ca^{2+} uptake. For **A** and **B**; number of experiments (or liver used) (n) = 7.

Şekil 2. Sıçan karaciğer mikrozoamlarında tapsigargininle indüklenmiş Ca^{2+} hareketleri. **A**, 1 mg/ml mikrozoam (M) ilavesi sonucu Ca^{2+} alınımının başlaması ve 6. dakikadaki 64 nM tapsigarginin (TGC^a) ilavesi oklarla gösterilmiştir. Deponun tamamen boşaltımı A23187 (1 $\mu\text{g}/\text{ml}$) ilavesiyle sağlanmış olup, ok ile gösterilmiştir. **B**, **A** ile aynıdır, bununla birlikte Ca^{2+} alınımının başlamasını takiben tapsigarginin komplementer miktarları (TGC^b ; 1.6 nM, TGC^c ; 8 nM, TGC^d ; 16 nM, TGC^e ; 38.4 nM) eklenmiştir. **A** ve **B** için; deney sayısı (ya da kullanılan karaciğer sayısı) (n) = 7'dir.

Inhibitory effect of TGC on Ca^{2+} -ATPase activity:

The ability of TGC to release nearly all sequestered Ca^{2+} and completely inhibit Ca^{2+} loading of rat liver microsomes (Figure 2) suggests that TGC may inhibit ER Ca^{2+} -ATPase activity. Figure 4 shows TGC inhibits Ca^{2+} -ATPase activity in rat liver microsomes. The potency is similar to the potency of stimulation of Ca^{2+} release, with 50 % inhibition of Ca^{2+} -stimulated ATPase by < 50 nM TGC (Figure 4).

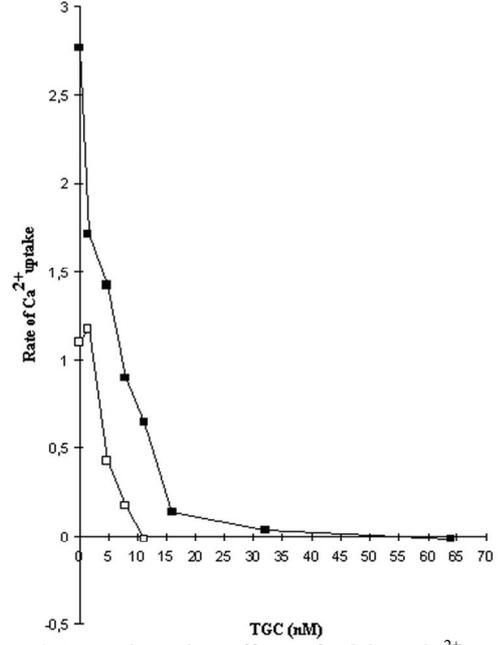


Figure 3. Dose-dependent effects of TGC on Ca^{2+} uptake from microsomes. In figure, (■) represents the rate of Ca^{2+} release from rat liver endoplasmic reticulum vesicles (1.12 mg/ml) for a range of 0-64 nM TGC concentration. (□), the same as (■), but amount of microsomes used were 0.56 mg/ml with the range of TGC from 0-16 nM. For □ and ■; number of experiments (or liver used) (n) = 7.

Şekil 3. Mikrozoamlardan Ca^{2+} alınımı üzerine TGC'nin doza bağımlı etkileri. Şekilde, 0-64 nM arası değişen farklı TGC konsantrasyon aralıkları için endoplazmik retikulum veziküllerinden (1.12 mg/ml) Ca^{2+} salınımı hızı (■) ile gösterilmiştir. (□), (■) ile aynıdır, ancak kullanılan mikrozoam 0.56 mg/ml olup, TGC konsantrasyonu ise 0-16 nM aralığında değişmektedir. □ ve ■ için; deney sayısı (ya da kullanılan karaciğer sayısı) (n) = 7'dir.

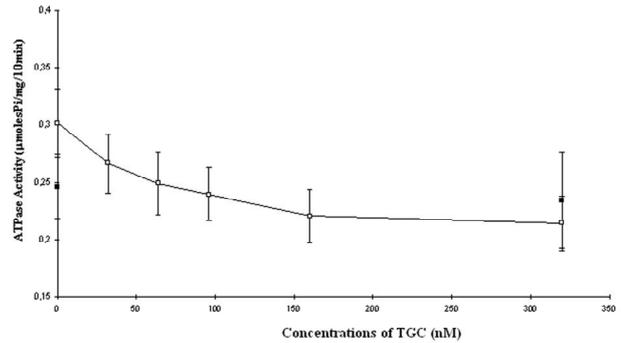


Figure 4. Inhibition of Ca^{2+} -ATPase activity by TGC in the presence (□) and absence (■) of Ca^{2+} . In figure, (□) represents the Ca^{2+} -ATPase activity of rat liver microsomes for a range of 0-320 nM thapsigargin (TGC) concentration in the presence of Ca^{2+} . (■), the same as (□), but in the absence of Ca^{2+} in the assay medium. Each value represents the Mean \pm S.D. For □; number of experiments (or liver used) (n) = 7 and for ■; n = 4. Şekil 4. Ca^{2+} -ATPaz aktivitesinin Ca^{2+} 'un varlığında (□) ve yokluğunda (■) TGC ile inhibisyonu. Şekilde, Ca^{2+} varlığında sıçan karaciğer mikrozoamlarındaki Ca^{2+} -ATPaz aktivitesi 0-320 nM arası değişen farklı TGC konsantrasyon (TGC) aralıkları için (□) ile gösterilmiştir. (■), (□) ile aynıdır, ancak ortamda Ca^{2+} yoktur. Her bir değer Ortalama \pm Standard Sapma'yı temsil etmektedir. □ için; deney sayısı (ya da kullanılan karaciğer sayısı) (n) = 7 ve ■ için; n = 4'tür.

Discussion and Conclusion

The network of Ca^{2+} channels and pumps permits the movement of Ca^{2+} between the cytoplasmic space and the extracellular fluid and between the cytoplasmic space and intracellular Ca^{2+} stores. As a result of this arrangement a fine control of $[\text{Ca}^{2+}]_i$ is obtained by Ca^{2+} -ATPases.

On the other hand, the inhibitors of intracellular Ca^{2+} -ATPases constitute a quite interesting and useful group of chemical compounds inasmuch as they provide important means of probing the catalytic and transport mechanism of these transport enzymes, their expression and regulation in cells and the role of cytosolic Ca^{2+} stores in cell biology.

Thapsigargin was shown in a number of cell types to increase $[\text{Ca}^{2+}]_i$ by inducing a rapid and dose-dependent Ca^{2+} release from intracellular Ca^{2+} stores without hydrolysis of phosphoinositides (26, 28). From the findings of Cullen et al. (7) and Caspersen and Treiman (4), it has been suggested that thapsigargin (TG) discharges intracellular stores by an acute and highly specific inhibition of the SERCA ATPases at subnanomolar concentrations (15). Therefore, a number of laboratories have reported that thapsigargin specifically inhibits SERCA ATPases (17, 25, 27) with little or no effect on the Ca^{2+} -ATPases of hepatocyte or erythrocyte PMCA (23).

The finding of a highly potent inhibition of the Ca^{2+} -activated microsomal ATPase indicates that the molecular target for thapsigargin is the Ca^{2+} -accumulation system rather than the efflux system. A similar dose dependency of the thapsigargin-induced Ca^{2+} release and pump inhibition, together with the ability of ATP depletion to elicit an identical release, provides strong evidence for identification of the ER Ca^{2+} ATPase as the site of thapsigargin action.

Very recently, the effect of TGC on Ca^{2+} movements has been investigated by using both digitonin-permeabilized and intact L1210 mouse lymphoma cells (22). It has been found that the TGC behaves exactly similar to TG in these cells too.

From the structural similarities between TGC and TG, their origination from the same *Thapsia* genus and by the results of our current study, it is just a conclusion that in this system (microsomes) TGC behaves like TG. TGC has the ability to increase the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by discharging the intracellular Ca^{2+} stores and causing a potent and specific inhibition of the ER Ca^{2+} -ATPase.

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