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# Thapsigargin inhibits voltage-activated calcium channels in adrenal glomerulosa cells

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Thapsigargin, an inhibitor of the microsomal  $\text{Ca}^{2+}$  pumps, has been extensively used to study the intracellular  $\text{Ca}^{2+}$  pool participating in the generation of the agonist-induced  $\text{Ca}^{2+}$  signal in various cell types. A dual effect of this agent was observed in bovine adrenal zona glomerulosa cells. At nanomolar concentrations, thapsigargin stimulated a sustained  $\text{Ca}^{2+}$  influx, probably resulting from  $\text{Ca}^{2+}$ -store depletion. In contrast, when added at micromolar concentrations, thapsigargin prevented the rise in cytosolic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_c$ ) induced by  $\text{K}^+$ . This inhibitory effect of thapsigargin on voltage-activated  $\text{Ca}^{2+}$  channels was confirmed by measuring  $\text{Ba}^{2+}$  currents by the

patch-clamp technique. Both low-threshold (T-type) and high-threshold (L-type)  $\text{Ca}^{2+}$  channels were affected by micromolar concentrations of thapsigargin. Analysis of the current–voltage relationship for T-type channels revealed that thapsigargin did not modify the sensitivity of these channels to the voltage, but decreased the maximal current flowing through the channels. In conclusion, thapsigargin appears to exert a dual effect on adrenal glomerulosa cells. At lower concentrations, this agent induces a sustained  $\text{Ca}^{2+}$  entry, whereas at higher concentrations it decreases  $[\text{Ca}^{2+}]_c$  by blocking voltage-activated  $\text{Ca}^{2+}$  channels.

## INTRODUCTION

Aldosterone biosynthesis in adrenal zona glomerulosa cells is precisely modulated by changes in the cytosolic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_c$ ) induced by activators such as  $\text{K}^+$  or angiotensin II (AngII), an octapeptide hormone [1–3]. Whereas  $\text{K}^+$  is believed to affect  $[\text{Ca}^{2+}]_c$  exclusively by activating voltage-sensitive  $\text{Ca}^{2+}$  channels [4,5], the mode of action of AngII is more complex. Indeed, AngII binding to its receptor is linked to the formation of diacylglycerol, an activator of protein kinase C [6], and of  $\text{Ins}(1,4,5)\text{P}_3$ , which is responsible for  $\text{Ca}^{2+}$  release from intracellular stores [7,8]. This initial phase of the  $\text{Ca}^{2+}$  signal in response to the hormone is followed by a sustained  $\text{Ca}^{2+}$ -entry phase [9], partially due to the activation of voltage-sensitive  $\text{Ca}^{2+}$  channels [10,11] and partially through a pathway directly regulated by intracellular  $\text{Ca}^{2+}$ -store depletion, also termed ‘capacitative’  $\text{Ca}^{2+}$  entry [12,13].

Thapsigargin, a sesquiterpene lactone tumour promoter, has been extensively used to characterize this latter type of  $\text{Ca}^{2+}$  influx in non-excitable cells [14,15]. Thapsigargin acts by blocking the microsomal  $\text{Ca}^{2+}$  pumps necessary for  $\text{Ca}^{2+}$  sequestration in intracellular pools, and therefore mimics the hormone by depleting  $\text{Ca}^{2+}$  stores, but without elevation of  $\text{Ins}(1,4,5)\text{P}_3$  [16]. An activation of  $\text{Ca}^{2+}$  entry by thapsigargin has been observed in various cell types, a finding that provided a strong support for the capacitative- $\text{Ca}^{2+}$ -entry hypothesis [17].

In bovine adrenal glomerulosa cells, the AngII-responsive and thapsigargin-sensitive  $\text{Ca}^{2+}$  pools are largely coincident, but a component of the agonist-induced  $\text{Ca}^{2+}$  influx, probably involving voltage-activated  $\text{Ca}^{2+}$  channels, cannot be mimicked by thapsigargin [13]. A similar effect of thapsigargin on  $[\text{Ca}^{2+}]_c$  has been described in rat glomerulosa cells [18], in which this agent exerts a steroidogenic action. In these cells the effects of AngII and thapsigargin on aldosterone production were additive, whereas the response to  $\text{K}^+$  was potentiated by thapsigargin.

Interestingly, in these cells, micromolar concentrations of thapsigargin markedly decreased the rise in  $[\text{Ca}^{2+}]_c$  induced by  $\text{K}^+$  [19].

In the present study we report that, in bovine adrenal glomerulosa cells, thapsigargin, in addition to increasing  $[\text{Ca}^{2+}]_c$  through a mechanism of influx activated by the depletion of intracellular  $\text{Ca}^{2+}$  stores, inhibits voltage-activated  $\text{Ca}^{2+}$  channels.

## MATERIALS AND METHODS

### Materials

Thapsigargin was obtained from Anawa (Zurich, Switzerland). Hepes, insulin, transferrin, sodium selenite, tetrodotoxin, ATP and GTP were purchased from Sigma. Dispace (Grade II) was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.), Percoll from Pharmacia (Piscataway, NJ, U.S.A.), and ascorbate from Merck (Darmstadt, Germany). Horse serum, fetal-calf serum (FCS) and Dulbecco’s modified Eagle medium (DMEM) were from Gibco (Grand Island, NY, U.S.A.). Metirapone was purchased from Aldrich (Milwaukee, WI, U.S.A.), and fura-2 and 1,2-bis-(2-aminophenoxy)ethane- $\text{NNN}'\text{N}'$ -tetra-acetic acid tetracaesium salt ( $\text{Cs}_4\text{BAPTA}$ ) were from Molecular Probes (Eugene, OR, U.S.A.).

### Isolation and culture of bovine glomerulosa cells

Bovine adrenal glomerulosa cells were prepared by enzymic dispersion with Dispace and purified on a Percoll density gradient, as described in detail elsewhere [20]. Cells were then plated on small glass coverslips in antibiotics-containing DMEM supplemented with 1 mM ascorbate, 1  $\mu\text{g}/\text{ml}$  insulin, 1  $\mu\text{g}/\text{ml}$  transferrin, 1 ng/ml sodium selenite, 5  $\mu\text{M}$  metirapone, 2 mM glutamine, 2% (v/v) FCS and 10% (v/v) horse serum, and incubated overnight at 37 °C in 5%  $\text{CO}_2$ . The next day, the medium was removed and replaced with serum-free DMEM, until the cells were used for patch-clamp experiments.

Abbreviations used:  $[\text{Ca}^{2+}]_c$ , cytosolic free  $\text{Ca}^{2+}$  concentration; AngII, [Ile5]angiotensin II; FCS, fetal-calf serum; DMEM, Dulbecco’s modified Eagle medium;  $\text{Cs}_4\text{BAPTA}$ , 1,2-bis-(2-aminophenoxy)ethane- $\text{NNN}'\text{N}'$ -tetra-acetic acid tetracaesium salt.

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### Measurement of $[Ca^{2+}]_i$

This was done in cell populations with the fluorescent probe fura-2. Freshly prepared cells were purified on Percoll density gradients, washed twice and resuspended in a Krebs-Ringer medium [20] at a concentration of  $10^7$  cells/ml, and then incubated at 37 °C for 30 min in the presence of 2  $\mu$ M fura-2 acetoxymethyl ester. The dye excess was then washed away, and the cells were kept at ambient temperature in the same medium. Batches of  $2 \times 10^6$  cells were sedimented just before use, and resuspended in 2 ml of Krebs-Ringer medium, in a thermostatically maintained cuvette at 37 °C. Fura-2 fluorescence (excitation at 340 nm and emission at 505 nm) was recorded in a Perkin-Elmer LS-3 fluorescence spectrometer, and  $[Ca^{2+}]_i$  was calibrated as previously described for quin2 [21], by using a value of 224 nM for the  $K_d$  of fura-2.

### Patch-clamp measurements

The activity of voltage-activated  $Ca^{2+}$  channels was recorded under voltage clamp in the whole-cell configuration of the patch clamp technique, essentially as described in [20]. The bath solution contained (in mM): 117 tetraethylammonium chloride, 20  $BaCl_2$ , 0.5  $MgCl_2$ , 5 D-glucose, 32 sucrose and 200 nM tetrodotoxin, and was buffered to pH 7.5 with 10 mM Hepes/CsOH. The patch pipette (Clark 150T) contained (in mM): 85 CsCl, 10 tetrabutylammonium chloride, 6  $MgCl_2$ , 5 sodium ATP and 0.04 GTP, and pH was buffered to 7.2 with 20 mM Hepes/CsOH. The pipette solution also contained 0.9 mM  $CaCl_2$  and 11 mM  $Cs_4BAPTA$  in order to buffer free  $Ca^{2+}$  below 50 nM and to stabilize the low access resistance from pipette to cell. Glomerulosa cells were voltage-clamped at a holding potential of -90 mV and depolarized as indicated in the legends of the Figures. The  $Ba^{2+}$  currents were filtered and automatically leak-subtracted as indicated elsewhere [20].

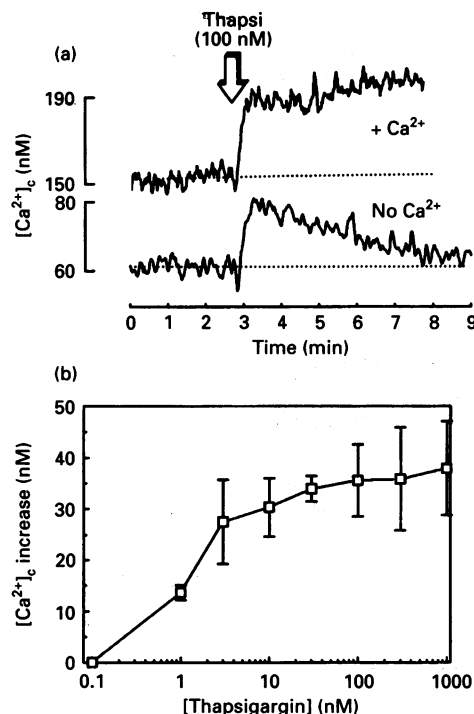
## RESULTS AND DISCUSSION

### Increase in $[Ca^{2+}]_i$ induced by thapsigargin

Addition of thapsigargin to fura-2 loaded bovine adrenal glomerulosa cells led to a small but reliable increase in  $[Ca^{2+}]_i$  (Figure 1a, upper trace). This  $Ca^{2+}$  response was sustained for more than 10 min and depended on the presence of extracellular  $Ca^{2+}$  (lower trace), a hallmark for  $Ca^{2+}$  influx. In addition, the response was totally unaffected by nifedipine (1  $\mu$ M), a dihydropyridine blocking the response to  $K^+$  (results not shown). The effect of thapsigargin was concentration-dependent, with a maximum observed at approx. 50 nM (Figure 1b). These results are in good agreement with those of Ely et al. [13] and confirm the presence in glomerulosa cells of a  $Ca^{2+}$ -entry pathway, although of minor importance, which is controlled by the depletion of intracellular  $Ca^{2+}$  stores [14] rather than by the membrane potential.

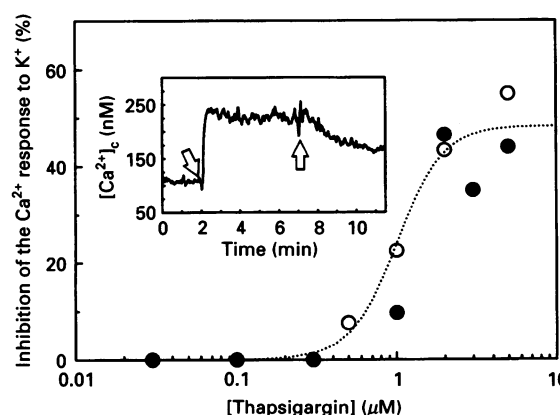
### Inhibition by thapsigargin of $K^+$ -induced $Ca^{2+}$ influx

Surprisingly, thapsigargin, at micromolar concentrations, markedly decreased the  $[Ca^{2+}]_i$  increase evoked by  $K^+$  (Figure 2, inset). The  $IC_{50}$ , approx. 1  $\mu$ M thapsigargin, did not appear to depend on  $K^+$  concentration in the range 6–25 mM (results not shown). The extent of inhibition by thapsigargin varied slightly from one cell preparation to the other, but no inhibition was observed at concentrations below 0.5  $\mu$ M (Figure 2). When thapsigargin was introduced in the medium before  $K^+$ , the



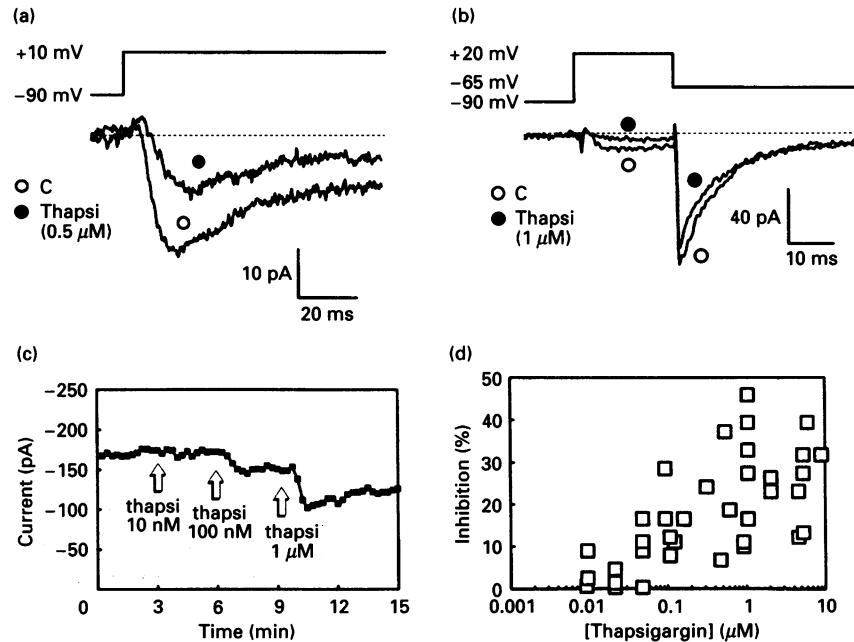
**Figure 1** Effect of thapsigargin on  $[Ca^{2+}]_i$ .

Bovine adrenal glomerulosa cells were isolated and loaded with fura-2 as described in the Materials and methods section. (a)  $Ca^{2+}$  response from cells exposed to 100 nM thapsigargin (Thapsi), added at the time indicated by the arrow, in the presence (upper trace) of 1.2 mM extracellular  $Ca^{2+}$ , or in the absence (lower trace) of added  $Ca^{2+}$  (1.2 mM EGTA). (b) The concentration-dependent increase in  $[Ca^{2+}]_i$  induced by thapsigargin in the presence of extracellular calcium was determined by measuring the value of  $[Ca^{2+}]_i$  before and 4–5 min after thapsigargin addition. Results are means  $\pm$  S.E.M. from five independent experiments.



**Figure 2** Inhibition by thapsigargin of the  $K^+$ -induced  $Ca^{2+}$  response

Fura-2-loaded cells were sequentially exposed to 9 mM ( $\circ$ ) or 12 mM ( $\bullet$ )  $K^+$  and then to thapsigargin (0.03–5  $\mu$ M). Inset: an example of the response to 9 mM  $K^+$  added after 2 min (first arrow) and its inhibition by 5  $\mu$ M thapsigargin (added at the time indicated by the second arrow). The decrease in  $[Ca^{2+}]_i$  was measured 3–5 min after thapsigargin addition and expressed as the percentage inhibition of the maximal response to  $K^+$ . Results are presented as a function of thapsigargin concentration and are the mean values from two to five determinations and from six different cell preparations. The dotted line describes a logistic function of the type:  $f(x) = A/[1 + (IC_{50}/x)^k]$ , where  $A$  is the maximal amplitude and  $k$  the slope factor, fitted to all individual data, with an  $IC_{50}$  of 1.01  $\mu$ M.



**Figure 3** Inhibition by thapsigargin of voltage-activated  $\text{Ba}^{2+}$  currents

Voltage-activated  $\text{Ba}^{2+}$  currents were recorded by the patch-clamp technique, in the whole-cell configuration, as indicated in the Materials and methods section. (a) Superimposed traces of  $\text{Ba}^{2+}$  currents elicited by a depolarization of the cell from  $-90$  to  $+10$  mV, before ( $\circ$ ; C) and 2 min after ( $\bullet$ ) addition of thapsigargin (Thapsi;  $0.5 \mu\text{M}$ ). The dotted line indicates the zero current level. (b) Same as (a), but this cell was depolarized for 20 ms to  $+20$  mV, and then repolarized to  $-65$  mV to evoke tail currents. The decay of the current was described by a single exponential function, with time constants of 9.14 and 9.95 ms for control and treated cells respectively. The first 2 ms recording after repolarization (during the capacitive transient and L-channel deactivation) was not included for curve fitting. (c) Time course of tail-current inhibition by thapsigargin. Slowly deactivating (T-type) currents were evoked every 15 s, as described in (b), and the initial current was extrapolated by fitting the decaying current to an exponential function and plotted as a function of time. Increasing concentrations of thapsigargin were added at the times indicated by the arrows. (d) Concentration-dependent effect of thapsigargin on T-type current. The percentage tail-current inhibition was estimated by averaging the current over a short period before addition of thapsigargin, and after inhibition had occurred. Data collected from 18 cells were plotted as a function of thapsigargin concentration.

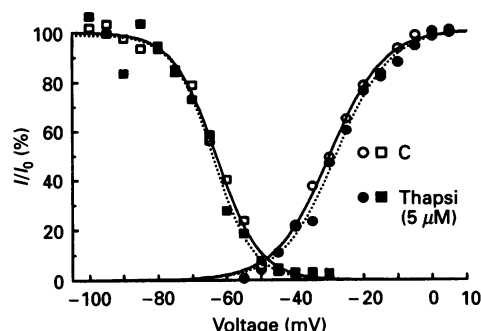
subsequent response to  $\text{K}^+$  was considerably decreased, but the sensitivity to  $\text{K}^+$ , as assessed by successive stimulations with increasing concentrations of the cation, remained unchanged (results not shown). These observations strongly suggested that thapsigargin affected  $\text{Ca}^{2+}$  channels opened by  $\text{K}^+$ .

#### Inhibition of voltage-activated $\text{Ca}^{2+}$ channels by thapsigargin

The action of thapsigargin on voltage-activated  $\text{Ca}^{2+}$  channels was directly assessed by the patch-clamp technique. Inward  $\text{Ba}^{2+}$  currents were evoked by depolarizing the cell from a holding potential of  $-90$  mV to  $+10$  mV. As described elsewhere [20,22], these currents developed to a maximum in less than 20 ms, and then rapidly decayed to a plateau which remained elevated for several hundred ms (Figure 3a, control trace). These biphasic kinetics are believed to reflect the activation of both low-threshold (transient, T-type), and high-threshold (long-lasting, L-type) channels [22]. Addition of  $0.5 \mu\text{M}$  thapsigargin to the bath resulted in a marked decrease in the current elicited by depolarization (Figure 3a). Thapsigargin affected both the peak and the plateau of the  $\text{Ba}^{2+}$  current, suggesting that both T- and L-type channels are sensitive to this agent.

Although the effect of thapsigargin appeared more pronounced on L-type currents (Figure 3a), we have focused our attention on T-type currents, because these currents are activated by physiological concentrations of  $\text{K}^+$  and are believed to be responsible for activation of steroidogenesis in glomerulosa cells [5]. In order to isolate specifically the effect of thapsigargin on T-type channels, slowly deactivating tail currents were induced upon repolar-

ization of the cell to  $-65$  mV after a short activation period at  $+20$  mV. These slowly deactivating currents are almost exclusively due to T-type  $\text{Ca}^{2+}$  channels [5]. An example of such a large tail current is presented in Figure 3(b). The current appeared immediately upon repolarization of the cell and decayed with a time constant of about 9 ms, which is a value characteristic of T-type currents in glomerulosa cells [5,20]. As expected, addition of a micromolar concentration of thapsigargin clearly decreased the size of the tail current (Figure 3b). To determine the inhibition of the current by a given concentration of the drug, the same voltage protocol as that described in Figure 3(b) was repeatedly performed every 15 s. At each pulse of voltage, the current was recorded, and the initial value of the decaying current was extrapolated after fitting the data with an exponential function. The value of the current at the moment of cell repolarization was then plotted as a function of time. An example of the time course of the effect of thapsigargin, added sequentially at increasing concentrations, is presented in Figure 3(c). Whereas a low concentration of thapsigargin (10 nM) did not affect the tail-current value, higher concentrations of this agent (100 nM or  $1 \mu\text{M}$ ) rapidly and markedly decreased its magnitude. The percentage inhibition of the current at various thapsigargin concentrations was similarly determined from 18 independent cells exposed to one or several thapsigargin concentrations and is shown in Figure 3(d). In spite of the wide dispersion of the results, which may reflect some cellular heterogeneity in the sensitivity to thapsigargin, it is possible to estimate a maximal inhibitory effect of this agent at around  $1 \mu\text{M}$ . This value, which is of the same order of magnitude as the concentration necessary



**Figure 4** Lack of effect of thapsigargin on T-channel sensitivity to voltage

Voltage-dependent activation and inactivation of T-type channels were measured, as described in [5], before (○, □; C) and after (●, ■) treatment with thapsigargin (Thapsi; 5 μM). The activation curves (○, ●) were determined by measuring slowly deactivating tail currents elicited by repolarization of the cell to -65 mV after a 20 ms channel activation at various test voltage amplitudes (-55 to +5 mV), whereas the steady-state inactivation curves (□, ■) were obtained by measuring the same current (at -65 mV) after maximal depolarization (+20 mV, 20 ms) from various conditioning pre-pulse potentials (-100 to -30 mV) lasting 10 s. Data were fitted to Boltzman's equation and normalized to the maximum of the function ( $I_0$ ). Each data point is the mean value from three different cells in which currents have been determined before and after addition of thapsigargin.

for inhibition of the  $\text{Ca}^{2+}$  response to  $\text{K}^+$ , appears much higher than the thapsigargin concentration responsible for maximal  $\text{Ca}^{2+}$  influx (Figure 1b). This suggests that both events, i.e.  $\text{Ca}^{2+}$  entry by the 'capacitative' pathway and inhibition of voltage-activated  $\text{Ca}^{2+}$  channels, are totally unrelated.

### Mechanism of T-type channel inhibition

The effect of thapsigargin on the voltage sensitivity of T-type channels was investigated by measuring the voltage dependency of channel activation and inactivation in the presence and in the absence of the drug. Activation was determined by application of 20 ms depolarizing test pulses to the indicated potentials (-55 to +5 mV) from a negative holding potential (-90 mV). Upon repolarization, tail currents were elicited and measured as described in the legend of Figure 3(b). Data were then plotted as a function of the activation potential, fitted to Boltzman's equation and normalized to the maximum of the current (Figure 4, circles). Correspondingly, the dependence of channel inactivation on voltage was determined by holding the cell at various potentials (-100 to -30 mV) for 10 s, leading to steady-state channel inactivation, before applying a strong depolarizing pulse (+20 mV) for 20 ms to open the remaining available channels. Similarly, tail currents were elicited upon repolarization, measured and plotted as a function of holding (inactivation) potential, before being fitted and normalized to Boltzman's equation (Figure 4, squares). The results show that thapsigargin (5 μM), although decreasing the maximal elicitable current by approx. 40% (results not shown), did not change the dependence of T-type channels on voltage. Indeed, no shift of the activation or inactivation curves of the channel could be observed after treatment with thapsigargin, as is the case, for example, with atrial natriuretic peptide [5] or nitrendipine [11]. In this regard,

the mechanism of T-channel inhibition by thapsigargin is comparable with that of tetrandrine, a recently described blocker of these channels [20], and will require further investigation to be resolved.

In conclusion, thapsigargin appears to exert a dual effect on adrenal glomerulosa cells. At nanomolar concentrations, this agent induces a sustained  $\text{Ca}^{2+}$  entry, presumably resulting from depletion of intracellular  $\text{Ca}^{2+}$  stores. At higher concentrations, thapsigargin inhibits voltage-activated  $\text{Ca}^{2+}$  channels, and therefore decreases the  $\text{K}^+$ -induced  $\text{Ca}^{2+}$  response. Whether this latter effect of thapsigargin is also present in other cell types remains to be confirmed. Recently, Vercesi et al. [23] have reported that thapsigargin, at concentrations above 10 μM, causes collapse of the mitochondrial membrane potential in *Trypanosoma brucei* and in rat liver, resulting in  $\text{Ca}^{2+}$  release from this organelle. These results therefore highlight the need for caution in interpreting the action of high concentrations of this agent on cellular  $\text{Ca}^{2+}$  homeostasis.

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