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3,5-Di-*t*-butyl-4-hydroxyanisole (DTBHA) activation of rat skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase

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Abstract

3,5-Di-*t*-butyl-4-hydroxyanisole (DTBHA) increased in a concentration-dependent manner (calculated pEC_{50} = 4.55 ± 0.18 M) the oxalate-stimulated Ca^{2+}-pumping rate of rat skeletal muscle sarcoplasmic reticulum (SR) vesicles. Kinetic analysis of this effect suggested that the activation of SR Ca^{2+}-ATPase operated by (DTBHA) was of both mixed and non-competitive type with respect to ATP in the range of concentrations 0.1–0.5 mM and above 1 mM, respectively; furthermore, it was independent of the free Ca^{2+} concentrations. This indicated that the enzyme activation took place through the acceleration of the enzyme-substrate complex breakdown. Moreover, it appeared that its target site was cyclopiazonic acid sensitive. The uncommon ability of (DTBHA) to upregulate SR Ca^{2+} uptake is of interest in view of its possible use for treating pathological conditions characterised by cell Ca^{2+} overload as well as genetic disorders where SR Ca^{2+} homeostasis is altered.

Keywords: 3,5-Di-*t*-butyl-4-hydroxyanisole; Sarcoplasmic reticulum Ca^{2+} homeostasis; Cyclopiazonic acid

1. Introduction

The availability in the cytosol of free Ca^{2+} is determined by Ca^{2+} release and uptake by intracellular Ca^{2+} store sites such as SR† and mitochondria, as well as by Ca^{2+} influx and efflux across cell membrane. The Ca^{2+}-ATPase in the intracellular Ca^{2+} store sites acts as an ion pump that transports Ca^{2+} from cytoplasm into the vesicular lumen to maintain a high gradient of Ca^{2+} across the membrane. The characteristics of this Ca^{2+} pump have been extensively investigated in skeletal muscle SR because of their well-developed structure [1–4] and their suitability for an in-depth analysis of Ca^{2+} transport mechanisms.

Ca^{2+}-ATPase of skeletal muscle SR catalyses the most important step in muscle relaxation by coupling cleavage of ATP to transport of two Ca^{2+} into the lumen of SR [5,6]. This process is associated with an ordered sequence of elementary events consisting of phosphorylation and dephosphorylation reactions of the enzyme as well as a conformational oscillation that exposes its Ca^{2+} binding moiety either at the cytoplasmic or the luminal side.

A dysfunction of SR Ca^{2+}-ATPase and/or Ca^{2+} channel has been proposed as a contributing factor to the development of cardiovascular diseases such as genetic hypertension [7], ischemia and reperfusion injury, myocardial stunning, and heart failure [8], as well as skeletal muscle diseases such as Brody's disease, myotonic dystrophy [9], and Duchenne muscular dystrophy [10].

A limited number of substances are reported in the literature that specifically modulate the Ca^{2+} pumping activity of SR and most of them act as inhibitors. Phenolic lipophilic antioxidants such as 3,5-di-*t*-butyl-4-hydroxytoluene (BHT) [11], 2,5-di-*t*-butyl-1,4-benzohydroquinone (BHQ) [12] and some naturally occurring compounds such as thapsigargin [13] inhibit the SR Ca^{2+}-ATPase. However, certain plant derivatives (e.g. gingerol and ellagic acid) [14] as well as jasmone and the synthetic compound, disulfiram [15,16], are known to stimulate Ca^{2+} pumping activity at both skeletal and cardiac muscles SR. These activators, therefore, represent valuable lead molecules for the development of new drugs potentially useful in human diseases characterised by pathological reduction in SR Ca^{2+}-ATPase activity or concentration. In fact, the unphysiologically high concentration of cytosolic Ca^{2+} observed in patients suffering

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†*Abbreviations:* BHQ, 2,5-di-*t*-butyl-1,4-benzohydroquinone; BHT, 3,5-di-*t*-butyl-4-hydroxytoluene; CPA, cyclopiazonic acid; DTBHA, 3,5-di-*t*-butyl-4-hydroxyanisole; and SR, sarcoplasmic reticulum.

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from such pathologies (see above) might be treated with these activators thus favoring the restoration of Ca^{2+} concentration after muscle stimulation.

We have previously characterized a series of sterically hindered phenols for their antioxidant properties in addition to their myorelaxing properties in gut smooth muscles [17]. Among them, DTBHA was proposed as a lead compound for the design of new drugs of high antioxidant and Ca^{2+}-blocking capabilities, thus potentially offering dual protection against tissue damage from ischemia-reperfusion injury. More recently, DTBHA myorelaxing activity on gastric as well as vascular smooth muscles has been analyzed in details [18–20]. In the former tissue, however, DTBHA-induced relaxation was antagonized by CPA, an inhibitor of the SR Ca^{2+}-ATPase [21]. Therefore, it was hypothesized that DTBHA activates the SR Ca^{2+}-ATPase, speeding up the removal of Ca^{2+} from the cytosol and thus promoting muscle relaxation. Ca^{2+} handling studies were therefore undertaken to analyze the effects of DTBHA on skeletal muscle SR. We investigated the ATP-dependent Ca^{2+} uptake in the presence of oxalate (a Ca^{2+} precipitating anion), by monitoring extra vesicular Ca^{2+} concentration directly with arsenazo III.

2. Materials and methods

2.1. Preparation of SR vesicles

SR vesicles, free from mitochondrial contamination, were prepared from rat skeletal muscle, as described by Goeger *et al.* [21]. In brief, muscle from the hind leg was minced with scissors and homogenized in 3 vols. of 0.1 M KCl, 2 mM EDTA, and 5 mM phosphate, pH 7.0, for 5 min with a Polytron, followed by 1 min in a Waring blender. The homogenate was centrifuged at 6,500 *g* for 15 min; the supernatant fraction was filtered through cheese cloth and centrifuged at 10,000 *g* for 15 min, and the SR vesicles were pelleted at 45,000 *g* for 1 h. The pellet was suspended by hand with a Dounce tissue grinder in a volume of 0.6 M KCl, 10% sucrose and 5 mM histidine, pH 7.0, equal to 40% of the initial tissue weight (v/w). After a 30-min incubation, the suspension was centrifuged at 10,000 *g* for 15 min, and the SR vesicles were pelleted at 45,000 *g* for 1 h, resuspended in 10% sucrose and 5 mM histidine, pH 7.0, equal to 20% of the initial tissue weight (v/w), and repelleted as above. The final pellet was suspended in the sucrose-histidine buffer in a volume equal to 5% of the tissue weight, divided into aliquots, and frozen at −80° until use. All isolation procedures were performed at 4°.

Protein was determined according to Bensadoun and Weinstein [22].

The average SR protein concentration employed in the assays was 75 μg/mL. Considering that the SR ATPase accounts for 60% of the protein in the preparation and assuming a M_r of 100 kDa [5,6], an enzyme concentration of 0.45 μM was thought to be present in the assay.

2.2. Measurement of Ca^{2+} uptake

Oxalate-facilitated Ca^{2+} uptake by SR vesicles was followed by dual-wavelength spectrophotometry using the Ca^{2+} indicator dye, arsenazo III, to monitor the external Ca^{2+} concentration. SR vesicles were added to the buffer solution (see below for composition) containing 100 μM arsenazo III, at 24°. Uptake of Ca^{2+} was followed by measuring the absorbance in the wavelength pair of 660–700 nm ($\Delta A_{660-700}$), which made the measurements more selective toward Ca^{2+} [23], with the use of a Shimadzu UV-160 spectrophotometer (Columbia, MD, USA). The absorbance changes were a linear function of Ca^{2+} concentration up to 40 μM (data not shown). Under these experimental conditions, the addition of A23187 at the end of the experiment did not produce significant Ca^{2+} release, indicating that in the presence of oxalate no Ca^{2+} release can affect the measurements. Furthermore, in order to calibrate the changes in absorbance of arsenazo III with the concentration of Ca^{2+} in the reaction mixture, known concentrations of Ca^{2+} were added to the reaction mixture in the absence of SR.

The assay mixture (final volume 1 mL) contained: 31.5 μM $CaCl_2$ (as free Ca^{2+}, see below), 100 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 5 mM oxalate, and 50 mM MOPS-KOH buffer (pH 7.0).

Free Ca^{2+} concentrations were estimated by computations with use of the computer program EqCal (BioSoft) by taking into account pH, Mg^{2+}, and ATP concentrations, as described by Fabiato and Fabiato [24]. Final Ca^{2+} concentrations stated in the text indicate free Ca^{2+}.

2.3. Effect of DTBHA on SR Ca^{2+} uptake

The effect of DTBHA on Ca^{2+} uptake by SR vesicles was investigated by adding DTBHA at various concentrations or vehicle (DMSO) to the reaction buffer prior to the addition of SR, unless otherwise specified.

In another series of experiments, the effect of DTBHA on Ca^{2+} uptake by SR vesicles was analyzed at different concentrations of either ATP (0.1–4 mM) or Ca^{2+} (1.8–31.5 μM).

2.4. Materials

DTBHA and CPA (Sigma, St. Louis, MO, USA) were dissolved in DMSO. This vehicle did not affect Ca^{2+} uptake by SR vesicles at the maximum concentration used (0.4%, v/v).

2.5. Statistical analysis

Unless original traces are shown, values reported indicate means of *n* number of animals (indicated in parentheses). Curve fitting was performed by GraphPad Prism version 3.02 (GraphPad Software). Analysis of variance

followed by Dunnet's post-hoc test was performed by using GraphPad InStat version 3.05. P-values < 0.05 were considered significant.

The equilibrium dissociation constant for DTBHA-free enzyme combination (K_s^A) and that for the interaction of the drug with the enzyme-substrate complex (K_m^A) were calculated graphically, according to Dixon and Webb [25].

3. Results

3.1. Enhancement by DTBHA of SR Ca^{2+} uptake at steady state

Following an initial period (~30 s), the rate of Ca^{2+} uptake by SR vesicles loaded with oxalate was constant for 60–120 s before gradually ceasing (Fig. 1A). This was the consequence of extravesicular Ca^{2+} concentration run out since the consecutive addition of a further aliquot of Ca^{2+} gave rise to a similar picture of uptake (data not shown). Ca^{2+} uptake was increased by DTBHA either when added after the reaction had reached a constant rate or when it was already present in the reaction medium. Activation of Ca^{2+} uptake by DTBHA was concentration-dependent, with a calculated pEC_{50} of 4.55 ± 0.18 M ($N = 3$) (Fig. 1B).

3.2. Effect of DTBHA on SR Ca^{2+} uptake as a function of ATP concentration

The kinetics of DTBHA activation were analyzed with respect to ATP concentration. The Michaelis–Menten analysis of Ca^{2+} uptake rate against ATP concentration (Fig. 2A) showed two components, as previously reported in the literature [26,27], thus indicating the existence of a high-affinity (activatory) and a low-affinity (regulatory) binding sites. At ATP concentrations above 0.5 mM (i.e. the regulatory binding site), K_m (0.455 mM) was not affected by DTBHA while V_{max} increased from 62.4 (control) to 69.2, 84.6, and 116.3 nmols × min^{-1} × mg^{-1} with 3, 10, and 30 μM DTBHA, respectively. Thus, DTBHA shows a non-competitive activation pattern with respect to ATP in the range of concentrations above 0.5 mM. In the concentration range of 0.1–0.5 mM (i.e. the activatory binding site), V_{max} increased from 44.6 (control) to 57.7, 78.3, and 110.1 nmols × min^{-1} × mg^{-1}, whereas K_m decreased from 0.080 (control) to 0.065, 0.055, and 0.046 mM with 3, 10, and 30 μM DTBHA, respectively. Thus, DTBHA shows a mixed stimulation pattern with respect to ATP in the range of concentrations below 1 mM. These results are consistent with DTBHA activation of SR Ca^{2+} uptake of the "non-essential" type with characteristics typical of both competitive and non-competitive activation [25]. "Non-essential" type activation results when the activator combines to both the enzyme or the enzyme-substrate complex with different affinities (K_s^A and K_m^A, respectively). A general mechanism

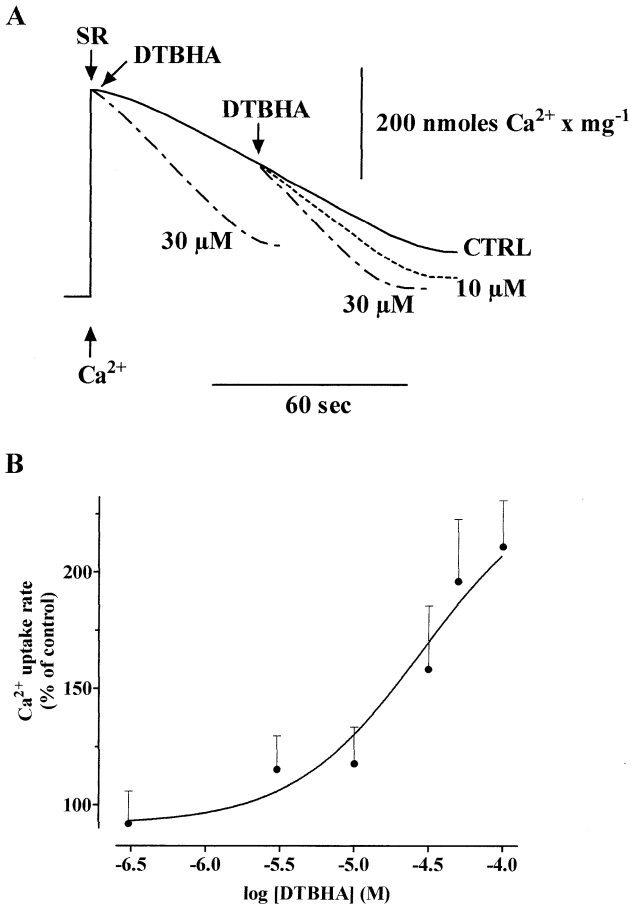

Fig. 1. Stimulatory effect of DTBHA on the Ca^{2+}-pumping activity of SR. SR vesicles were assayed for Ca^{2+} uptake by continuously monitoring the difference in absorbance ($A_{700-660}$) with arsenazo III at 24°. Ca^{2+} uptake was started by the addition of 75 μg/mL of SR vesicles. A) Traces representative of at least three experiments with different vesicles preparations, where DTBHA was either present into the reaction medium or added in mid course of Ca^{2+}-pumping process. The sharp increase in the trace corresponded to the addition of Ca^{2+} (to a final free concentration of 31.5 μM). CTRL: control. B) Concentration-dependent effect of DTBHA activation. Ordinate scale, response is reported as a percentage of the control (vehicle). Data points are mean values and vertical lines represent SEM ($N = 3$).

can be written as:

$$\begin{array}{ccccc} E & \xleftrightarrow{K_s^I} & ES & \xrightarrow{k} & E+P \\ \Updownarrow K_s^A & & \Updownarrow K_m^A & & \\ EA & \xleftrightarrow[K_s^I]{} & EAS & \xrightarrow[k']{} & E+A+P \end{array}$$

When plotting the reciprocals of the change in slope or intercept (Δ_{slope} or $\Delta_{intercept}$ obtained in the double reciprocal plot of the data in Fig. 2A, plot not shown) against the reciprocal of the activator concentration, a straight line was generated (Fig. 2B) and values for all the constants can be obtained. In the case of DTBHA, because K_m^A (9.7 μM) was about 3.6 times lower than K_s^A (34.6 μM), the primarily "uncompetitive" character of DTBHA action on SR Ca^{2+}-ATPase emerged.

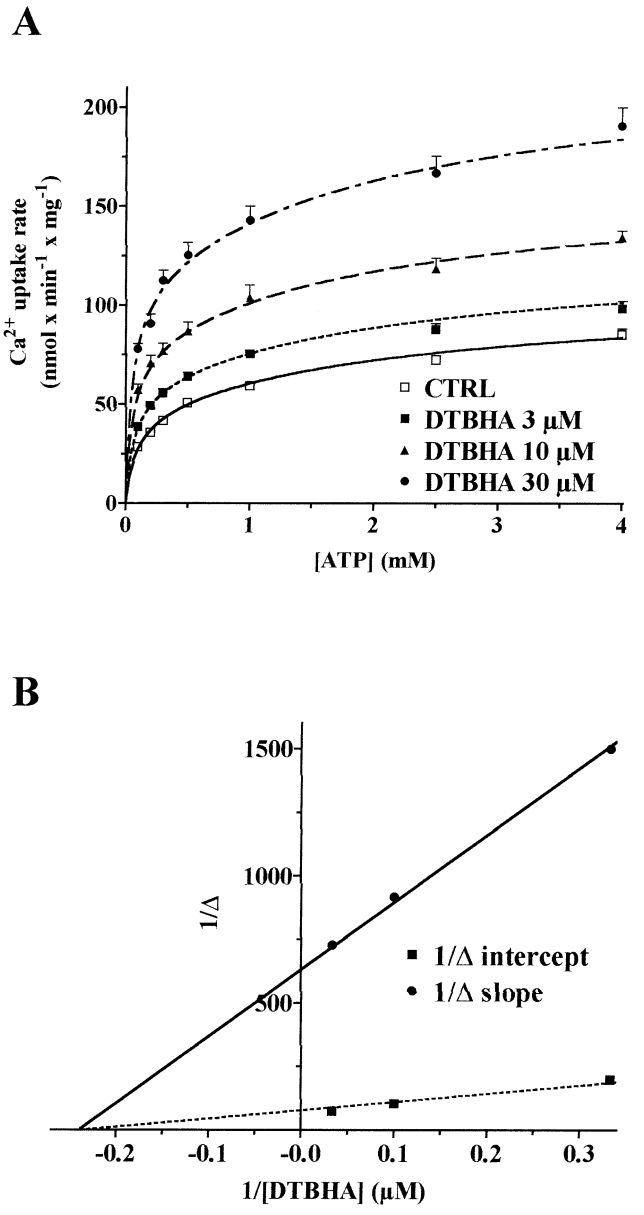

Fig. 2. Effect of DTBHA on the dependence of SR-Ca^{2+} ATPase Ca^{2+} uptake on the concentration of ATP. A) Michaelis–Menten plot showing the effect of DTBHA on the ATP concentration-dependence of SR Ca^{2+}-ATPase. SR vesicles were incubated in an assay medium whose ATP concentration varied between 0.1 and 4 mM. The $CaCl_2$ concentration was maintained at pCa 5.3. The points on the graph represent the mean values of 6 separate vesicles preparations ± SEM. Steady-state velocities were obtained from linear plots of Ca^{2+} uptake. CTRL: control. B) Plots of the 1/change in slope or intercept (1/Δ) against 1/activator relative to the high affinity (activatory) binding site. Δ_{slope} and $\Delta_{intercept}$ were determined by subtracting the values in presence of the activator from that in its absence obtained in the double reciprocal plot (not shown) of the data in Fig. 2A.

3.3. Effect of DTBHA on SR Ca^{2+} uptake as a function of Ca^{2+} concentration

In order to evaluate whether a change in affinity of the Ca^{2+}-ATPase may account for the effect of DTBHA on Ca^{2+} uptake, the dependence of Ca^{2+} transport on Ca^{2+} concentration was evaluated. Fig. 3 shows the dependence of Ca^{2+} uptake on log [Ca^{2+}] and how it was affected by DTBHA. At the various Ca^{2+} concentrations tested, Ca^{2+} uptake was increased by about 20, 60, and 100% in the presence of 3, 10, and 30 μM DTBHA, respectively. The data clearly showed that the potentiating effect of DTBHA was relatively unaffected by the external Ca^{2+} concentration over the range 1.9–31.5 μM.

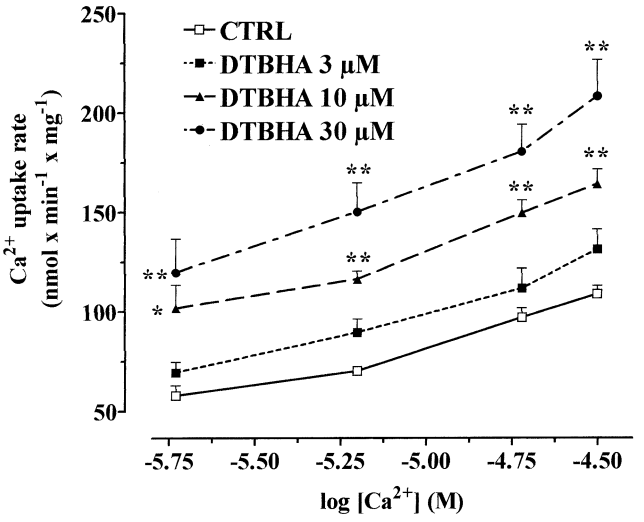

Fig. 3. Effect of DTBHA on Ca^{2+} dependence of SR-Ca^{2+}-ATPase Ca^{2+} uptake. Effect of DTBHA on SR Ca^{2+}-ATPase activity as a function of the concentration of free Ca^{2+} (1.9–31.5 μM). Skeletal muscle SR microsomes were incubated in the presence of 1 mM ATP and with 3–30 μM DTBHA or with the vehicle alone (CTRL). Each point represents the mean ± SEM of five experiments with different vesicles preparations. Ca^{2+} uptake was assayed in a similar way to that described in the legend to Fig. 1. *$P < 0.05$, **$P < 0.01$, Dunnett's post test.

3.4. Antagonism by DTBHA on CPA inhibition of SR Ca^{2+} uptake

The effect of DTBHA on Ca^{2+} uptake by SR was examined under the condition in which the Ca^{2+} pump was gradually blocked by increasing concentrations of CPA. As shown in Fig. 4, 50 μM DTBHA was able to revert to or even above control values the rate of Ca^{2+} uptake at CPA concentrations of 3 μM or lower, respectively. This suggests that DTBHA site of action was CPA sensitive.

4. Discussion

A long-lasting excess of free Ca^{2+} concentration in the cytosol is a serious threat to cell survival. In fact, a large rise of intracellular Ca^{2+} concentration, if prolonged for tens of seconds, results in irreversible damage of a number of cellular functions [28]. Under normal conditions, this event is prevented by a very fast and relatively homogeneous return of $[Ca^{2+}]_i$ to resting levels operated by cell organelle Ca^{2+} accumulation.

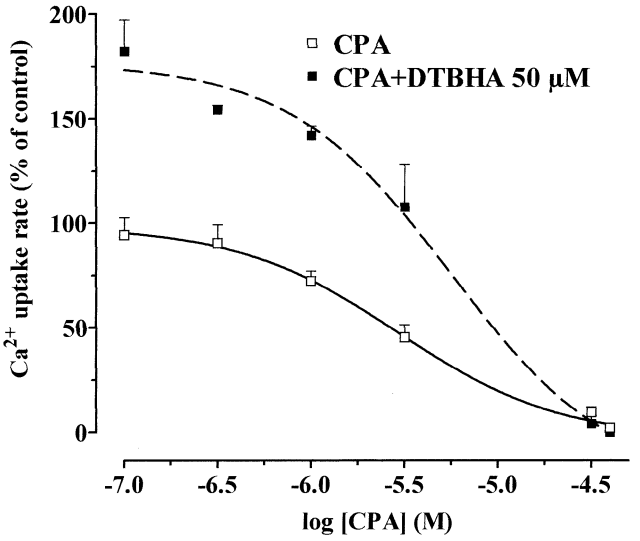

Fig. 4. Antagonism by DTBHA of CPA-inhibited Ca^{2+} transport in skeletal SR vesicles. Concentration-dependent inhibition of Ca^{2+}-uptake by CPA and its reversal by 50 μM DTBHA. Each point represents the mean ± SEM of three experiments with different vesicles preparations. Ca^{2+} uptake was assayed in a similar way to that described in the legend to Fig. 1.

The findings reported here show that DTBHA activates rat skeletal muscle SR Ca^{2+}-ATPase in a concentration-dependent manner pointing to DTBHA as a lead compound for the development of selective activators of Ca^{2+}-ATPase with possible therapeutic applications (see introduction) and as a potentially important tool for the scientific investigation of Ca^{2+} handling by skeletal muscle and other cells.

Sokolove *et al.* [11] reported that phenolic antioxidants (including DTBHA) inhibit at low extravesicular Ca^{2+} concentrations (0.3 μM) but enhance the Ca^{2+}-ATPase activity at high Ca^{2+} concentrations (>5 μM). The latter effect gains more importance in view of recent data collected by using fluorescent Ca^{2+} indicators which show that the concentration of cytosolic Ca^{2+} during pathological conditions may reach levels >5 μM [29].

SR influx and efflux of Ca^{2+} may occur simultaneously; therefore, an apparently increased accumulation of this ion might result from either stimulation of uptake or inhibition of Ca^{2+} extrusion. The latter, however, does not apply to this study, for two reasons: first, in the presence of oxalate, A23187 did not cause Ca^{2+} release from SR; second, DTBHA neither affected chlorocresol-induced Ca^{2+} release from SR [30] nor caused any Ca^{2+} release from vesicles previously loaded with Ca^{2+} (unpublished observations).

The kinetics of activation of DTBHA was examined with respect to ATP or free Ca^{2+}. The action of DTBHA on SR Ca^{2+}-ATPase appears principally due to an un-competitive high affinity interaction of the drug with the enzyme-substrate intermediate complex formed during the catalytic process, rather than to a competition with the substrate at the free active site of the enzyme. Nevertheless, the present result suggests that DTBHA may interact with both the catalytic and regulatory ATP-binding sites [26,31], although with different activation patterns, i.e. mixed and non-competitive, respectively. Therefore, activation by DTBHA of Ca^{2+} pumping activity even at low ATP concentrations (such as those occurring during hypoxia; for a review see [32]) might represent a valuable intervention in order to limit Ca^{2+} overload, e.g. immediately after ischemia, when the redistribution of intracellular Ca^{2+} [8] is due to the inhibition of both SR and sarcolemmal Ca^{2+}-ATPases owing to a reduced ATP availability, in spite of a persisting SR Ca^{2+} release.

The major effect of DTBHA on the Ca^{2+}-dependent activity of the skeletal SR was the result of an apparent increase in the rate constant for enzyme-substrate complex breakdown. It can be concluded that this is due to a direct interaction of DTBHA with the Ca^{2+}-ATPase. Thus, DTBHA activates SR Ca^{2+}-ATPase probably through the acceleration of product formation in a Ca^{2+} concentration range that is crucial for the pump activation under physiological conditions. However, further studies are required to define more precisely the molecular mechanism of DTBHA activation. Furthermore, the stimulatory effect of DTBHA was independent of the Ca^{2+} levels in the medium. In fact, the rate of Ca^{2+} uptake was increased at all [Ca^{2+}] tested. Thus, the effects of DTBHA should enhance the buffering rate of the cell under conditions of Ca^{2+} overload.

It is noteworthy that the stimulatory profile displayed by DTBHA on the SR Ca^{2+} pump is different from that reported for gingerol. In contrast to DTBHA, the activation by gingerol is uncompetitive and competitive with respect to ATP concentrations of 0.2–0.5 mM and above 1 mM, respectively, and mixed type with respect to free Ca^{2+} concentration [33]. However, gingerol was also reported to accelerate the enzyme-substrate complex breakdown.

An interesting matter of discussion arises when considering the structural characteristics of DTBHA and those of BHQ, a specific inhibitor of SR Ca^{2+}-ATPase [12]. The structural similarity of DTBHA and BHQ suggests that they function through different and specific mechanisms. Both compounds are antioxidants ([17] and unpublished observation) and possess similar redox potentials [34]. Thus, there is no relationship between the effects on the SR Ca^{2+}-ATPase and the antioxidant activity of the compounds. The differences in their structure (i.e. the position of the bulky *t*-butyl groups, and the number of hydroxyl groups), might be responsible for their different effects on the same target protein. Further information about the structure-activity relationship (currently under study in this laboratory) should help to clarify the mechanisms by which DTBHA and BHQ exert opposite effects on SR Ca^{2+} uptake.

In a previous paper, DTBHA myorelaxing activity on gastric fundus smooth muscle was shown to be antagonized by CPA [18]. The experiments presented here demonstrate that DTBHA-activation of SR Ca^{2+} uptake is also antagonized by CPA. Therefore, we can hypothesize that DTBHA, by activating the SR Ca^{2+}-ATPase and thus speeding up the

removal of Ca^{2+} from the cytosol, should facilitate muscle relaxation. Furthermore, it is interesting to note that DTBHA EC_{50} reported here is similar to that measured in rat ileum longitudinal smooth muscle [17]: this observation further supports the hypothesis that stimulation of Ca^{2+} removal into the SR can be the molecular mechanism by which the myorelaxing activity of DTBHA takes place.

In conclusion, stimulation of the SR Ca^{2+}-pump may presumably have little effect on the average cytoplasmic Ca^{2+} concentration at steady state, inasmuch the more rapid elimination of Ca^{2+} may be offset by a greater Ca^{2+} release from SR. Nevertheless, the role of DTBHA in modulating SR Ca^{2+} homeostasis as well as in scavenging radicals may be particularly beneficial for the maintenance of normal cell function in pathological conditions characterized by cytoplasmic accumulation of Ca^{2+} (or cell Ca^{2+} overload), as it may occur during oxidative stress and post-hypoxic/ischaemic-reperfusion. Experiments in progress at this laboratory support this hypothesis [35]. Finally, owing to its novel profile of activation, DTBHA represents an interesting tool to investigate the catalytic and transport mechanism of Ca^{2+} by the purified SERCA pumps.

Acknowledgments

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