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3,5-Di-*t*-butylcatechol (DTCAT) as an activator of rat skeletal muscle ryanodine receptor Ca^{2+} channel (RyRC)

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Abstract

In the present study, the effects of 3,5-di-*t*-butylcatechol (DTCAT) on ryanodine receptor Ca^{2+} channel (RyRC) of skeletal muscle sarcoplasmic reticulum (SR) vesicles were investigated, both by monitoring extravesicular Ca^{2+} concentration directly with the Ca^{2+} indicator dye arsenazo III and by studying the high-affinity [^{3}H]ryanodine binding. DTCAT stimulated Ca^{2+} release from junctional (terminal cisternae) vesicles in a concentration-dependent manner, with a threshold activating concentration of 30 μM and a pEC_{50} value of 3.43 ± 0.03 M. The release of Ca^{2+} induced by DTCAT was antagonized in a concentration-dependent manner by ruthenium red, thus indicating that RyRC is involved in the mechanism of stimulation. A structure–activity relationship analysis carried out on a limited number of compounds suggested that both hydroxy and *t*-butyl groups in DTCAT were important for the activation of RyRC. DTCAT inhibited [^{3}H]ryanodine binding to SR vesicles with a K_i of 232.5 μM, thus indicating that it acted directly at the skeletal muscle ryanodine receptor binding site to stimulate Ca^{2+} release. In conclusion, the ability of DTCAT to release Ca^{2+} from TC vesicles of skeletal muscle is noteworthy in view of its possible use as an alternative compound to either caffeine or halothane for performing the ''In vitro contracture test'' to diagnose the susceptibility of some patients to develop malignant hyperthermia under particular pharmacological treatments.

Keywords: 3,5-Di-*t*-butylcatechol; Ryanodine receptor Ca^{2+} channel; Rat skeletal sarcoplasmic reticulum

1. Introduction

The sarcoplasmic reticulum (SR) is a multifunctional signalling organelle that responds to surface membrane depolarisation, via the transverse tubule system, and releases and subsequently resequesters Ca^{2+}, thus playing a major role in regulating the skeletal muscle contraction–relaxation cycle [1]. This process is generally referred to as excitation–contraction coupling. The transient increase in $[Ca^{2+}]_i$ is achieved through the opening of Ca^{2+} release channel in the SR membrane [2], i.e. the ryanodine receptor Ca^{2+} channel (RyRC).

Ryanodine receptor Ca^{2+} channel was identified as the SR Ca^{2+} release channel about 15 years ago. The *RyRC* gene family, subsequently cloned [3], has been shown to exist as at least three distinct types in mammals, the muscular (skeletal) one being the type 1 [4,5].

Ca^{2+} release from intracellular stores triggers a variety of cell functions, the underlying mechanism thus representing a potential target for pharmacological interventions. In recent years, RyRC alterations have been implicated in the pathogenesis of several muscular diseases, such as malignant hyperthermia (MH) [6], central core disease (an autosomal-dominant human congenital myopathy) [7,8], myasthenia gravis [9], and fatigue [10,11]. MH susceptibility is an inherited autosomal disorder of skeletal muscle in which commonly used anaesthetics and depolarising muscle contracting agents can trigger an abnormally high release of Ca^{2+} from SR [12], thus causing a sustained, uncontrolled muscle contracture, hypermetabolism, hyperkalemia, hyperthermia, and cardiac arrhythmia that can be fatal unless the patient

Abbreviations: DTCAT, 3,5-di-*t*-butylcatechol; RyRC, ryanodine receptor Ca^{2+} channel; SR, sarcoplasmic reticulum; MH, malignant hyperthermia; TC, terminal cisternae; RR, ruthenium red; DIICAT, 3,5-diisopropylcatechol; DTBP, 2,4-di-*t*-butylphenol; TBC, 4-*t*-butyl catechol; DTHB, 3,5-di-*t*-butyl-2-hydroxy-benzaldehyde; CMC, 4-chloro-*m*-cresol; IVCT, in vitro contracture test

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is promptly treated with dantrolene or dantrolene-like drugs. To date, nine point mutations have been identified in human *RyRC* gene 1, which render biopsied muscles of patients carrying such *RyRC* gene 1 mutations hypersensitive to the contracture-triggering agents caffeine and halothane. This enhanced sensitivity is exploited in the in vitro contracture test (IVCT) to diagnose patients prone to MH. Although this IVCT is well standardized and characterized by a relatively high sensitivity and specificity, false negative results cannot be avoided which may cause serious risks to the concerned individuals. Therefore, improvement of the functional tests by additional procedures is needed.

A large number of chemically different substances have been reported to modulate Ca^{2+} release from isolated terminal cisternae (TC) vesicles via RyRC (for a review see [13]). Their actions are often complex and/or not completely understood and a basic distinction between agonists and antagonists is not tenable as several modulators may either stimulate or inhibit Ca^{2+} release, depending on concentration or incubation time, as well as on the interaction with different molecular sites of RyRC. Nevertheless, an attempt to classify both endogenous and exogenous modulators on the basis of their mechanism of action, rather than chemical structure, has been made [13]. These compounds, which include ions, nucleotides, lipid derivatives, enzymes as well as many drugs are important tools for studying intracellular Ca^{2+} homeostasis, and represent valuable probes for revealing patients prone to develop MH under particular circumstances (e.g. general anaesthesia with certain volatile agents [14]).

We have previously characterised a series of sterically hindered phenols for their antioxidant as well as their myorelaxing property in gut smooth muscles [15]. It was suggested that the hydroxy group on the benzene ring, hindered by a bulky lipophilic moiety, is a structural requirement that confers the compound a Ca^{2+}-antagonistic activity in smooth muscle cells [15–19]. More recently, Ca^{2+} handling (uptake and release) studies were undertaken to analyse the effects of these sterically hindered phenols on skeletal muscle SR. Among them, 2,6-di-*t*-butyl-4-methoxyphenol (DTBHA) was shown to activate rat skeletal muscle SR Ca^{2+}-ATPase, pointing to DTBHA as a lead compound for the development of selective activators of Ca^{2+}-ATPase with possible therapeutic applications [20]. Here we demonstrate that 3,5-di-*t*-butylcatechol (DTCAT) promotes the release of Ca^{2+} from a ruthenium red (RR)-sensitive Ca^{2+} release channel localized in TC vesicles and it inhibits [^{3}H]ryanodine binding to rat skeletal SR vesicles, as well. Moreover, a comparative analysis of some structural analogues of DTCAT never tested before in this system (see Table 1) has been performed to better understand the requirements for this action. The hypothesis that DTCAT acts at the skeletal muscle ryanodine receptor binding site to stimulate Ca^{2+} release is forwarded.

2. Materials and methods

2.1. Materials

Arsenazo III and RR were purchased from Sigma Chimica; Aldrich Chemical Co. has supplied DTCAT and 3,5-diisopropylcatechol (DIICAT); 2,4-di-*t*-butylphenol (DTBP), 4-*t*-butyl catechol (TBC), 3,5-di-*t*-butyl-2-hydroxy-benzaldehyde (DTHB) were from Lancaster Synthesis; 4-chloro-*m*-cresol (CMC) was obtained from Fluka Chemie AG; Calbiochem has supplied A23187 and ryanodine. [^{3}H]Ryanodine (specific activity 56.0 Ci/mmol) was purchased from NEN Life Science products. All other materials were of analytical grade or of the highest available grade. All reagents and agents were dissolved in MilliQ deionised water.

Stock solutions of ryanodine, DTCAT, DIICAT, TBC, DTBP, DTHB and CMC, dissolved in dimethylsulfoxide (DMSO), were stored at −20 °C. This vehicle did not affect either Ca^{2+} uptake/release by TC vesicles or [^{3}H]ryanodine binding at the maximum concentration used (0.9%, v/v).

2.2. Preparation of SR and TC vesicles

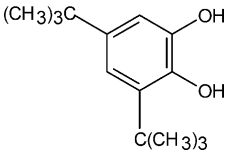
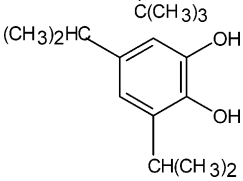
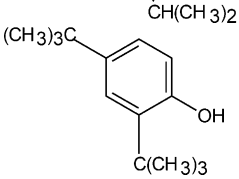
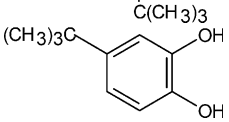
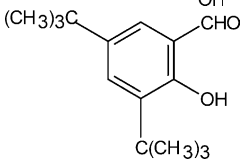
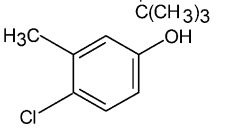
Male Sprague–Dawley rats (250–350 g; Charles River, Italia) were anaesthetized with a mixture of Ketavet® (Gellini) and Rompum® (Bayer), decapitated and bled. Hind leg skeletal muscle was immediately removed, cleaned of adhering fat and connective tissue and frozen at −80 °C. SR (for binding assay) and TC vesicles (for Ca^{2+} release assay), free from mitochondrial contamination, were prepared as described by Goeger et al. [21] and Saito et al. [22], respectively.

Protein was determined according to Bensadoun and Weinstein [23].

2.3. Measurement of Ca^{2+} release

Ca^{2+} release from isolated TC fractions was measured with a Shimadzu UV-160 spectrophotometer by monitoring the A_{660}–A_{700} ($\Delta A_{660-700}$) value of the Ca^{2+} indicator dye arsenazo III, by modifications of the method described by Palade [24], with pyrophosphate as an intravesicular Ca^{2+} sequestering anion. The use of pyrophosphate speeded up the rate at which SR can be loaded with Ca^{2+} and also allowed the accumulated Ca^{2+} to be released in response to agonists of the RyRC [24]. Ca^{2+} (12.5 μM final concentration) was added to the buffer solution (final volume 2 ml) containing 92.5 mM KCl, 1 mM ATP, 7.5 mM Na pyrophosphate, 100 μM arsenazo III, and 18.5 mM Mops–KOH buffer (pH 7.0), at 23 °C. When a steady-state value of optical density was reached, TC vesicles were added up to a final protein concentration of 0.1 mg/ml in order to establish their uptake capability. After a second addition of 50 μM Ca^{2+}, to complete Ca^{2+}

Table 1
Chemical structure of the compounds employed in this study and their effects on Ca^{2+} release from TC vesicles

Drug	Chemical structure	Ca^{2+} release (nmol/mg of protein)	
		300 μM	1 mM
DTCAT		184.3 ± 18.3	493.6 ± 24.8
DIICAT		116.6 ± 12.3	258.6 ± 12.5
DTBP		77.0 ± 6.2	148.2 ± 10.8
TBC		11.0 ± 2.0	32.9 ± 4.3
DTHB		9.3 ± 4.4	N.D.
CMC		N.D.	144.6 ± 35.2

CMC, 4-chloro-*m*-cresol; DIICAT, 3,5-diisopropylcatechol; DTBP, 2,4-di-*t*-butylphenol; DTCAT, 3,5-di-*t*-butylcatechol; DTHB, 3,5-di-*t*-butyl-2-hydroxybenzaldehyde; TBC, 4-*t*-butyl catechol. Data represent mean ± S.E.M. (n = 3–6). N.D.: not determined.

loading of vesicles, Ca^{2+} release was triggered by different compounds in the absence or presence of various concentrations of RR. At the end of each experiment, 2 μM A23187 was added to the cuvette, followed by 12.5 μM Ca^{2+} in order to calibrate the changes in absorbance of arsenazo III.

Free Ca^{2+} concentrations were calculated using the computer programme EqCal (BioSoft) by taking into account pH and ATP concentration, as described by Fabiato and Fabiato [25].

2.4. [^{3}H]Ryanodine receptor binding assay

[^{3}H]Ryanodine binding assay was performed according to the method of Simeoni et al. [26] with some modifications.

For saturation experiments, 50 μg of SR vesicles (final volume 0.2 ml) was incubated for 90 min at 36 °C in 10 mM Na-HEPES buffer (pH 7.2) containing 0.2 M KCl and 100 μM $CaCl_2$ (medium A) and either increasing concentrations of [^{3}H]ryanodine (0.5–5 nM) or a fixed amount of [^{3}H]ryanodine (5 nM) plus increasing concentrations of unlabelled ryanodine (2.5–50 nM). The incubation was terminated by rapid filtration on Whatman GF/B glass fibre filters, which were then washed three times with 5 ml of medium A-Ca^{2+} free and twice with 5 ml of 10% (v/v) ethanol. The filters were then placed in scintillation vials containing 5 ml of Ultima Gold^{TM} liquid scintillation, and the radioactivity measured in a LS5000CE ß-counter (Beckman Instruments).

The displacement of radiolabelled ryanodine was studied by using a fixed concentration (5 nM) of [^{3}H]ryanodine plus increasing concentrations of either DTCAT (0.01–5 mM) or ryanodine (0.001–2 μM). Non-specific binding was established by means of 10 μM unlabeled ryanodine. The assay was performed as described for saturation experiments. All the experiments were always run in triplicate.

2.5. Statistical analysis

Unless original traces are shown, values reported indicate means ± S.E.M. of n number of animals (indicated in parentheses). Curve fitting was performed by GraphPad Prism version 3.05 (GraphPad Software). Analysis of variance (ANOVA) followed by Dunnett's post hoc test or Student's t-test for paired samples were obtained using GraphPad InStat version 3.05 (GraphPad Software), as appropriate. P values <0.05 were considered significant.

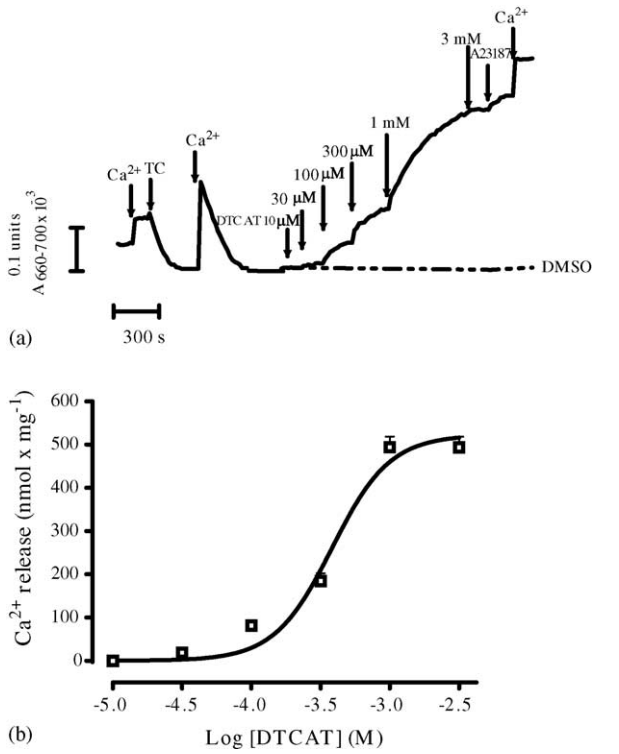


Fig. 1. DTCAT-induced Ca^{2+} release from TC vesicles. (a) Ca^{2+} (12.5 μM) decrease in the suspension solution started with the addition of 0.1 mg/ml TC vesicles at 23 °C in Mops–buffer. After uptake was completed, TC vesicles were loaded with a further addition of 50 μM Ca^{2+} and subsequently exposed to cumulative concentrations of DTCAT (0.01–3 mM). The final upward deflection at the end of the trace represents the further addition of 12.5 μM Ca^{2+}, in the presence of 2 μM A23187, made for calibrating the assay system. Upward deflections indicate Ca^{2+} release, downward deflections indicate Ca^{2+} accumulation. Trace, representative of experiments performed in quadruplicate, shows the difference in arsenazo III absorbance ($A_{660–700}$). Dashed trace represents the effect of vehicle. (b) Concentration-dependent effect of DTCAT activation of Ca^{2+} release. Line represents the best fitting of data points as determined by non-linear regression analysis. Data points are mean values and vertical bars represent S.E.M. ($n = 4–5$).

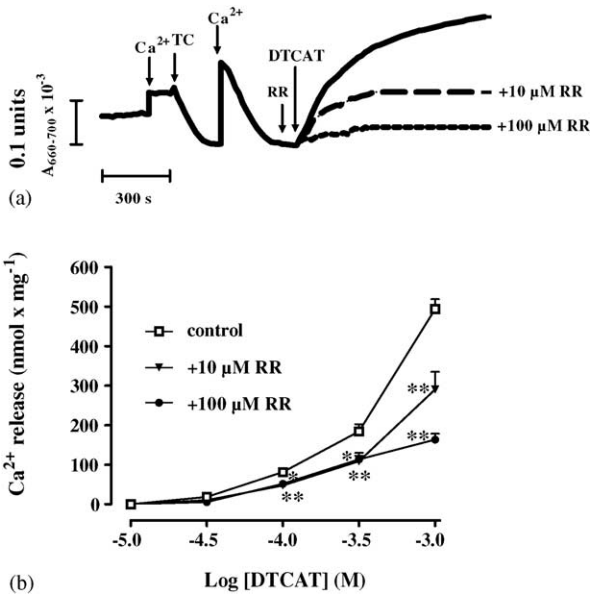


Fig. 2. Antagonism of DTCAT-induced Ca^{2+} release by RR. The experiments were carried out as described in the legend to Fig. 1. (a) RR (10 or 100 μM) was added 1 min before DTCAT (1 mM). Traces, representative of experiments performed in quadruplicate, show the difference in arsenazo III absorbance ($A_{660–700}$). (b) Concentration-dependent effect of DTCAT activation in the absence or presence of RR (10 or 100 μM). Data points are mean values and vertical bars represent S.E.M. ($n = 4–5$). $^{*}P < 0.05$, $^{**}P < 0.01$, Dunnett's post test.

Saturable binding constants relative to the binding of [^{3}H]ryanodine were calculated by using LIGAND program [27,28], while the IC_{50} value for DTCAT or ryanodine inhibition was obtained by plotting specific binding (% of control) versus the inhibitor concentration (M) and fitted with a non-linear (sigmoidal) analysis (GraphPad Prism 3.05). The K_i was calculated according to the method of Cheng and Prusoff [29].

3. Results

3.1. Characterisation of DTCAT-induced Ca^{2+} release from TC vesicles

Fig. 1 shows the concentration-dependent effect of DTCAT on the kinetics of Ca^{2+} release from TC vesicles actively loaded with Ca^{2+}. The Ca^{2+} uptake capability of TC vesicles pre-loaded with pyrophosphate, in the linear portion of the curve, amounted to about 90 nmol/mg of protein. After completion of Ca^{2+} loading, the addition of DTCAT (0.01–3 mM) caused a concentration-dependent release of Ca^{2+} (Fig. 1a) with a pEC_{50} value of 3.43 ± 0.03 M ($n = 4$), and a threshold activating concentration of 30 μM (Fig. 1b).

In another series of experiments, the ability of RR to inhibit Ca^{2+} release was checked to determine the pathway of DTCAT-induced Ca^{2+} release. As shown in Fig. 2a, 1 mM DTCAT triggered Ca^{2+} efflux from TC vesicles. RR (10 and 100 μM) significantly inhibited DTCAT-induced Ca^{2+} release in a concentration-dependent manner (Fig. 2a,b).

3.2. Effects of some DTCAT-analogues on Ca^{2+} release from TC vesicles

Four compounds related to DTCAT, namely DIICAT, DTBP, TBC and DTHB, were tested to try to ascertain the structural requirements for a compound to trigger Ca^{2+} release from TC vesicles. When in DTCAT the two *t*-butyl groups were replaced with two isopropyl groups, giving rise to DIICAT, the potency of the latter compound was half that of DTCAT (Fig. 3a). Abstraction of either one hydroxy or one *t*-butyl group gave rise to DTBP and TBC, respectively, which exhibited a much lower potency as compared to DTCAT (Fig. 3b) or null activity as was the case of TBC (Fig. 3c). In addition, the activity disappeared also when the 1-hydroxy was substituted by an aldehyde group, as it happens in DTHB (Fig. 3d). Both DIICAT-and

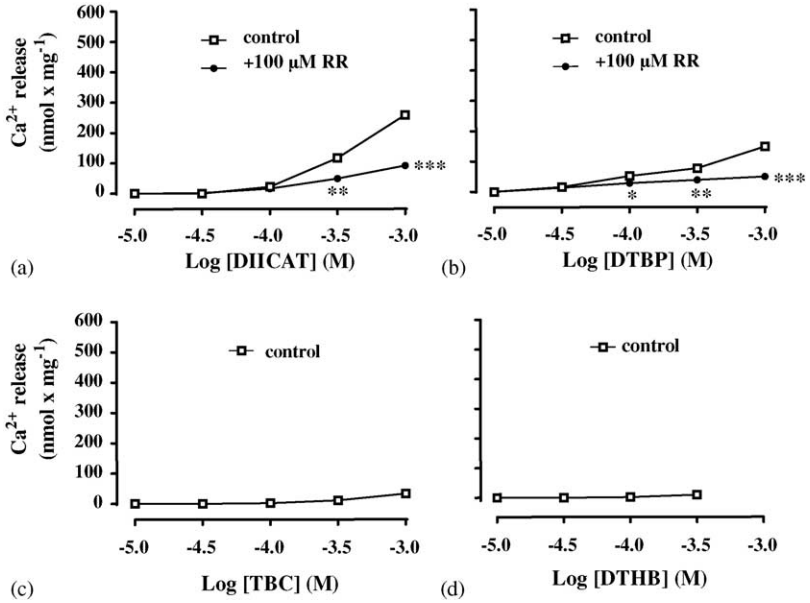


Fig. 3. Ca^{2+} releasing effect of some structural analogues of DTCAT. The experiments were carried out as described in the legend to Fig. 1. (a) DIICAT-, (b) DTBP-, (c) TBC-, (d) DTHB-induced Ca^{2+} release was tested in the absence or presence of 100 μM RR. Data points are mean values and vertical bars represent S.E.M. ($n = 3$–6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's t-test.

DTBP-induced Ca^{2+} release were significantly inhibited by 100 μM RR (Fig. 3a,b).

The effects on TC vesicles Ca^{2+} release sustained by DTCAT-related molecules, together with that of CMC, a known activator of RyRC [30], are compared in Table 1 at either a maximal or an intermediate effective concentration. Noticeably, under the same experimental conditions, DTCAT was over than three-fold more active than CMC.

3.3. Effect of DTCAT on [^{3}H]ryanodine binding to SR vesicles

Fig. 4 reports the saturation curve obtained by incubating 50 μg of SR vesicles with a mixture of hot/cold ryanodine varying from 0.5 to 50 nM final concentration. Ryanodine showed a dissociation constant (K_d) for RyRC of 15.2 nM and SR vesicles exhibited a maximal receptor density (B_{max}) of 0.851 pmol/mg.

Both ryanodine and DTCAT inhibited [^{3}H]ryanodine binding in a concentration-dependent fashion, with IC_{50} values of 13.9 nM and 310 μM that corresponded to K_i values of 10.4 nM and 232.5 μM, respectively (Fig. 5).

4. Discussion

It is well recognised that RyRC function is affected by endogenous substances as well as xenobiotics of various chemical nature (for a review see [13]). For the first time, DTCAT is shown here to trigger Ca^{2+} release from SR. Inhibition of DTCAT-induced Ca^{2+} release by RR as well

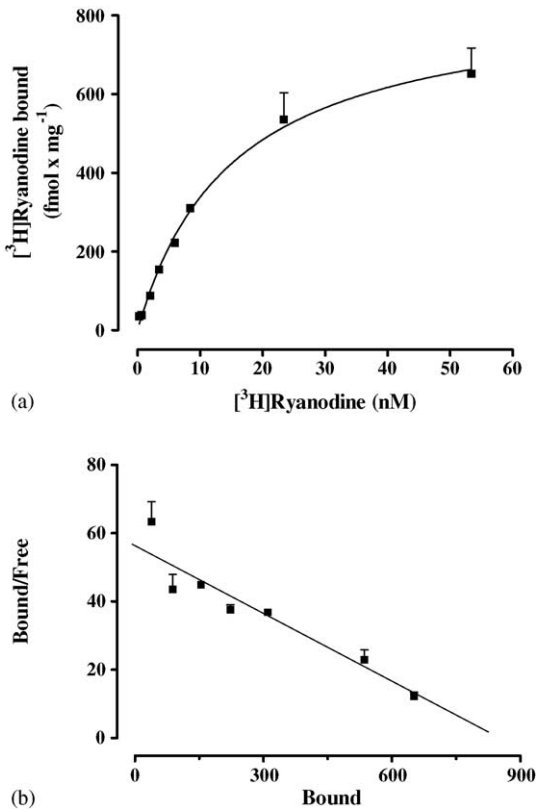


Fig. 4. Binding of [^{3}H]ryanodine to rat skeletal muscle SR vesicles. Depicted saturation curve (a) and Scatchard plot (b) were obtained as described in Methods section. Data points are mean values and vertical bars represent S.E.M. K_d and B_{max} values were 15.2 nM and 0.851 pmol/mg, respectively.

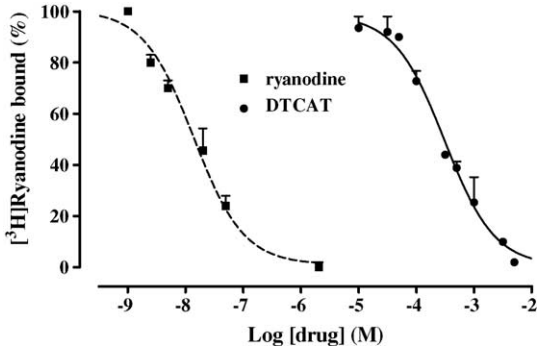


Fig. 5. Inhibition of specific binding of 5 nM [^{3}H]ryanodine to rat skeletal muscle SR vesicles by increasing concentrations of DTCAT and ryanodine. Line represents the best theoretical fitting of data as determined by non-linear least squares regression analysis. Data points are mean values and vertical bars represent S.E.M. from three experiments run in triplicate. K_i values, calculated according to the method of Cheng and Prusoff [29] by using K_d value reported in Fig. 4, were 232.5 μM (DTCAT) and 10.4 nM (ryanodine), respectively.

as binding experiments with [^{3}H]ryanodine clearly indicate that the effect of DTCAT on Ca^{2+} efflux stems from its interaction with RyRC. Ruthenium red, in fact, has been described as a specific blocker of this intracellular Ca^{2+} channel [31]. It must be underlined, however, that in the present investigation RR inhibition of DTCAT-elicited Ca^{2+} release was observed at relatively high concentrations, at variance with data reported in the literature (e.g. [30]) showing how RR prevents chemically induced Ca^{2+} release at concentrations one order of magnitude lower than those used in the present experiments. This might be due to the strong and specific interaction of DTCAT with RyRC, as demonstrated by the present binding data (see below).

[^{3}H]Ryanodine binding assay has been widely used to probe the functional state of RyRC [32]. Ligands that have been shown to activate or inhibit RyRC also modulate correspondently [^{3}H]ryanodine binding. Hence, we have investigated the possibility that DTCAT exerts its effect by acting as a modulator of the skeletal muscle RyRC using the [^{3}H]ryanodine binding assay. Surprisingly, this compound did not stimulate the binding of the labelled alkaloid to rat skeletal muscle SR vesicles, as was the case of other Ca^{2+} releasing compounds (e.g. caffeine and CMC [33]), but inhibited it with a K_i of 232 μM. These data suggest that DTCAT and CMC interact with different binding sites of RyRC, possibly acting through different mechanisms. Furthermore, our data indicate that DTCAT competes with ryanodine at its binding site on RyRC structure. Finally, it is worth noting that the EC_{50} value measured in the Ca^{2+} release assay (i.e. 372 μM) well correlates to the IC_{50} value measured in the binding assay (i.e. 310 μM). The potency of DTCAT, however, was four order of magnitude lower than that of the specific ligand ryanodine (IC_{50} = 13.9 nM).

Trying to clarify the structural requirements for DTCAT activation of Ca^{2+} release from TC vesicles, this study has been extended to a series of compounds structurally related to DTCAT (see Table 1). It was found that the presence of both the 1,2-hydroxy groups and 3,5-di-*t*-butyl groups is necessary for the activation of Ca^{2+} release via RyRC. DTCAT efficacy to induce Ca^{2+} release decreased markedly or even vanished when *t*-butyl hindrance on the 2-hydroxy group was reduced or removed, as it was the case with DIICAT and TBC, respectively. We can speculate that the Ca^{2+}-releasing potency of DTCAT is related to the reactivity of the 2-hydroxy group, in terms of its capability to donate a proton. The two *t*-butyl groups, which may facilitate DTCAT partition in a hydrophobic pocket of the membrane, in fact, are electron donors and most likely render more electronegative the hydroxy group at position 2 in the aromatic ring. It might be hypothesized that the electronegative hydroxy group of DTCAT interacts with a positively charged moiety in the RyRC gating domain. Finally, the hydroxy group at position 1 seems to be affected to a lesser extent by the two electron donor groups at position 3 and 5, i.e. it is less acidic; nevertheless, its presence is critical for DTCAT activity.

Recently CMC, a preservative, previously used as an additive of drug preparations to be administered intravenously, has been shown to activate specifically SR Ca^{2+} release in intact cell systems, in SR vesicles as well as at single channel level [33–35]. Since μM concentrations of CMC also induce contracture in muscles and potentiate the contracturing response to mM caffeine in MH-susceptible patients [36], CMC has been proposed as a tool to improve the effectiveness of the IVCT for detecting MH-prone patients [37,38]. Noteworthy, in the present study, the found threshold concentration for DTCAT was 30 μM, lower than that of CMC (75 μM) [38], thus suggesting a higher affinity of DTCAT for RyRC as compared to CMC.

In conclusion, DTCAT represents an interesting pharmacological and biochemical tool to investigate skeletal muscle RyRC-mediated Ca^{2+} release in physiological and/or pathological conditions.

Acknowledgements

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