Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 69 (2005) 485–491
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

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

Fabio Fusi*, Donata Iozzi, Giampietro Sgaragli, Maria Frosini

Dipartimento di Scienze Biomediche, Università degli Studi di Siena, via A. Moro 2, 53100 Siena, Italy

Received 16 September 2004; accepted 27 October 2004

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

* Corresponding author. Tel.: + 39 0577 234438; fax: +39 0577 234446.
E-mail address: fusif@unisi.it (F. Fusi).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.10.013

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+}