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3,5-Di-*t*-butyl catechol is a potent human ryanodine receptor 1 activator, not suitable for the diagnosis of malignant hyperthermia susceptibility

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ABSTRACT

3,5-Di-*t*-butyl catechol (DTCAT) releases Ca^{2+} from rat skeletal muscle sarcoplasmic reticulum (SR) vesicles. Hence, it is a candidate for use as a substitute for halothane or caffeine in the *in vitro* contracture test for the diagnosis of susceptibility to malignant hyperthermia (MH).

To characterize the effect of DTCAT at cell level, Ca^{2+} release experiments were performed on cultured, human skeletal muscle myotubes using the fluorescent Ca^{2+} indicator fura2-AM. DTCAT was also assayed in the *in vitro* contracture test on human skeletal muscle bundles obtained from individuals diagnosed susceptible (MHS), normal (MHN) or equivocal for halothane (MHEH) and compared to the standard test substances caffeine and halothane.

DTCAT increased, in a concentration-dependent manner and with a higher efficacy as compared to caffeine, the free, intracellular Ca^{2+} levels of cultured MHN and MHS skeletal muscle myotubes. This effect was similar in both types of myotubes and involved the release of Ca^{2+} from SR stores as well as Ca^{2+}-influx from the extracellular space. Inhibition of ryanodine receptors either with ryanodine or with ruthenium red markedly reduced DTCAT-induced increase in intracellular Ca^{2+} concentration while abolishing that induced by caffeine. In MHN skeletal muscle bundles, DTCAT induced contractures with an EC_{50} value of 160 ± 91 μM. However, the sensitivity of MHS or MHEH muscles to DTCAT was similar to that of MHN muscles.

In conclusion, DTCAT is not suitable for the diagnosis of MH susceptibility due to its failure to discriminate between MHN and MHS muscles.

1. Introduction

3,5-Di-*t*-butyl catechol (DTCAT) has been demonstrated to promote the release of Ca^{2+} from a ruthenium red-sensitive store localized in terminal cisternae vesicles and to inhibit [^{3}H]ryanodine binding to sarcoplasmic reticulum (SR) vesicles of rat skeletal muscle, thus implying a direct activation of the SR Ca^{2+} release channel, the ryanodine receptor 1 [1]. Substances acting directly on the ryanodine receptor can be valuable probes for detecting susceptibility to malignant hyperthermia (MH), an inherited, life-threatening anaesthesia-related disorder of Ca^{2+} homeostasis in human skeletal muscle. MH is triggered by increased intracellular Ca^{2+} levels in skeletal muscle cells upon exposure to substances such as volatile anaesthetics and the neuromuscular blocking drug succinylcholine [2]. Vigorous exercise, stress [3] and even fever [4] may promote MH-like episodes, although very rarely. In most cases of MH, mutations of the gene encoding for ryanodine receptor 1 render the channel susceptible to being activated by the aforementioned triggering substances [5].

Currently, the ryanodine receptor 1 activators caffeine and halothane are used for the diagnosis of MH susceptibility by means of the standardized *in vitro* contracture test [6,7] performed on human dissected muscle bundles. If both test substances induce a sustained muscle contracture at critical concentrations, the individual is diagnosed susceptible to MH (MHS). When both substances fail to induce muscle contractures, the patient is considered non susceptible (MHN). When muscles react to only one of the test substances, the diagnosis is equivocal (MHEH, for halothane; MHEC,

Abbreviations: DTCAT, 3,5-di-*t*-butyl catechol; SR, sarcoplasmic reticulum; MH, malignant hyperthermia; MHS, malignant hyperthermia susceptible; MHN, malignant hyperthermia normal; MHE, malignant hyperthermia equivocal; MHEH, malignant hyperthermia equivocal for halothane; MHEC, malignant hyperthermia equivocal for caffeine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; EGTA, ethylene glycol tetraacetic acid; DMSO, dimethylsulphoxide.

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for caffeine) and patients are clinically regarded MHS. The *in vitro* contracture test is very sensitive in identifying MHS but it has only a limited specificity within 78% (North American protocol) and 94% (European Protocol; [8]). This means that some of the MHS – and particularly the MHE – may be false positive. A positive MH diagnosis is a lifelong burden to the person concerned as an *in vitro* contracture test is usually not repeated, entailing problems for both the patient and his/her family. As MHS is a hereditary condition, testing of all relatives is strongly recommended. Moreover, anaesthesiologists perform general anaesthesia in an MHS individual only after careful planning and preparation of a volatile anaesthetic-free anaesthesia machine [9]. Therefore, trying to clarify equivocal results and thus increasing the specificity of the *in vitro* contracture test would be mandatory in anaesthesiology.

The ability to release Ca^{2+} from SR makes DTCAT a possible alternative test substance for the *in vitro* contracture test. The ideal test substance should be able to release Ca^{2+} from ryanodine sensitive stores with a much higher efficacy in MHS than in MHN muscles thus clearly discriminating MHS from MHN patients and preventing equivocal results. Since the action of DTCAT on intact human skeletal muscle has not been investigated before, we assessed its ability both to release Ca^{2+} in primary human skeletal muscle myotubes with the Ca^{2+} imaging technique, and to induce contractures in human skeletal muscle bundles.

2. Materials and methods

The study was approved by the ethics committee of the Medical University of Vienna, Austria. Surplus muscle bundles obtained during the muscle biopsy for the *in vitro* contracture test were used after the patient informed consent.

2.1. Human skeletal muscle cells culture

Human muscle bundles (100–350 mg) were used for skeletal muscle cell culture, as already described [10]. In brief, muscle bundles were freed from fat and connective tissue, cut and digested with collagenase (type IA, 0.5 mg/ml) and trypsin (0.25 mg/ml) in a Ca^{2+}- and Mg^{2+}-free phosphate buffered salt solution. After centrifugation ($200 \times g$), the cell suspension was washed, filtered, and finally seeded in growth medium in 50 ml cell culture flasks. Growth and differentiation media were prepared according to [10].

Cells were kept in an incubator at 37 °C under humidified atmosphere containing 2.5% CO_2, grown close to confluence, and reseeded on 25 mm glass cover slips coated with fibronectin or matrigel. In an incubator under air containing 5% CO_2, adherent cells were exposed to differentiation medium for 4–8 days in order to promote the fusion of myogenic satellite cells into myotubes (Fig. 1).

2.2. Determination of intracellular Ca^{2+} concentration

Myotubes were incubated with the Ca^{2+}-sensitive, fluorescence dye fura2-AM. The loading buffer was Tyrode's solution (concentrations in mM: 137 NaCl, 5.6 glucose, 5.4 KCl, 2.2 $NaHCO_3$, 1.1 $MgCl_2$, 0.4 NaH_2PO_4, 10 HEPES/Na, 1.8 $CaCl_2$, pH 7.4) supplemented with 2.5–5 μM fura2-AM and 0.025% pluronic F-127. After incubation in loading buffer for 45–60 min at 37 °C had been performed, excess dye was washed out and coverslips were placed into the perfusion chamber of a Nikon fluorescence microscope (Nikon, Vienna, Austria) equipped with a 40× objective. Imaging experiments were performed at room temperature in Tyrode's solution containing 1.8 mM Ca^{2+}, in nominally Ca^{2+} free Tyrode's solution ($Ca^{2+} = 0$) with no $CaCl_2$ added, or in Tyrode's solution adjusted to 88 nM Ca^{2+} with 1 mM EGTA and 0.7 mM $CaCl_2$. Only multinucleated myotubes which reacted to the HK depolarizing solution with a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were used for experiments, assuming a normal skeletal muscle-specific, excitation–contraction coupling mechanism. Usually but not necessarily myotubes showed contractility upon depolarization; only rarely they contracted spontaneously. The HK depolarizing solution consisted of a nominally Ca^{2+}-free Tyrode's solution containing 60 mM KCl and 80 mM NaCl. Fluorescence intensity was monitored at the emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm with use of a VisiTech monochromator (Sunderland, UK).

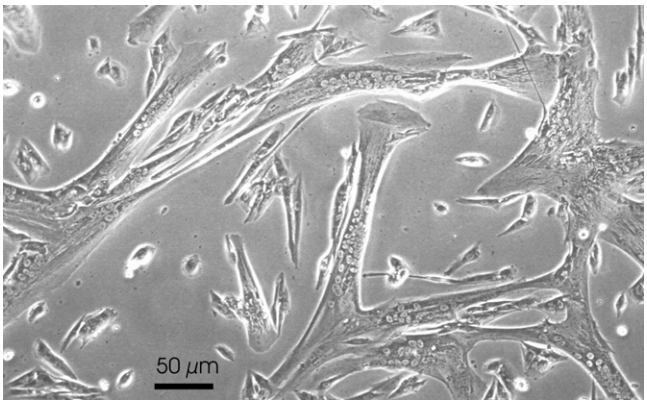

Fig. 1. Micrography of myotubes. Human myocytes were grown in a proliferation promoting medium until a density of approximately 70% was achieved. Then the medium was changed to differentiation medium to promote the fusion to multinucleated myotubes. These differentiated cells were used for experiments to determine the effects of DTCAT on intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$).

Stored images (sample interval, 0.5–2 s) were analyzed using the QC2000 software package (VisiTech, Sunderland, UK). Resting $[Ca^{2+}]_i$ was defined by the average of the last ten data points before application of any substance. The $[Ca^{2+}]_i$ transient value was determined by the peak value reached within the application time of a substance. To calculate $[Ca^{2+}]_i$ values, calibration of fura2 fluorescence signals was done according to [11]. The parameters of the Grynkiewicz-equation were obtained by the use of the pentapotassium salt of fura2 (5 μM) in a solution mimicking the intracellular milieu [12].

Patch pipettes were used to dialyse myotubes with a solution containing 100 μM ruthenium red for blocking ryanodine receptor 1. Seal formation was assessed under voltage clamp conditions with an Axopatch 1D amplifier connected to an Axon Digidata 1200 AD/DA board and a PC equipped with pClamp6 data acquisition software (Molecular Devices Corporation, CA, USA). Resistance of patch electrodes (PG10150-4, WPI, Sarasota, FL, USA) ranged from 0.8 to 2 MΩ. Intracellular solution contained (concentrations in mM): 125 CsOH, 125 aspartic acid, 15 CsCl, 5 EGTA/Na, 1 $MgCl_2$, 5 ATP/K_2, and 5 Na_2 creatine phosphate. CsOH was used to adjust pH to 7.2. Myotubes were superfused with Tyrode's solution. After seal formation, the voltage clamp was switched off.

Influx of external Ca^{2+} was demonstrated by Mn^{2+} quenching of fura2 fluorescence, at the isosbestic wavelength for fura2. The slope of the fluorescence decay, measured by linear regression analysis either in the absence or presence of either DTCAT or caffeine, served as an indirect measure for Ca^{2+} entry into the cell. In the presence of 1.8 mM extracellular Ca^{2+} and 500 μM $MnCl_2$, the fluorescence intensity was monitored at 340, 360 and 380 nm, allowing the simultaneous determination of $[Ca^{2+}]_i$ and Ca^{2+} influx.