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R(+)-pulegone impairs Ca^{2+} homeostasis and causes negative inotropism in mammalian myocardium

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ABSTRACT

The present study aimed to investigate the inotropic effects of R(+)-pulegone, a monoterpene found in plant species belonging to the genus *Mentha*, on the mammalian heart. In electrically stimulated guinea pig atria, R(+)-pulegone reduced the contractile force (~83%) and decreased the contraction time measured at 50% of the maximum force amplitude (CT_{50}) from 45.8 ± 6.2 ms to 36.9 ± 6.2 ms, suggesting that R(+)-pulegone may have an effect on Ca^{2+} homeostasis. Nifedipine (40 μM), taken as a positive control, showed a very similar profile. To explore the hypothesis that R(+)-pulegone is somehow affecting Ca^{2+} handling, we determined concentration–response curves for both $CaCl_2$ and BAY K8644. R(+)-pulegone shifted these curves rightward. Using isolated mouse ventricular cardiomyocytes, we measured whole-cell L-type Ca^{2+} current and observed an $I_{Ca,L}$ peak reduction of 13.7 ± 2.5% and 40.2 ± 2.9% after a 3-min perfusion with 0.11 and 1.1 mM of R(+)-pulegone, respectively. In addition, the intracellular Ca^{2+} transient was decreased (72.9%) by 3.2 mM R(+)-pulegone, with no significant changes in $[Ca^{2+}]_i$ transient decay kinetics. Moreover, R(+)-pulegone at 1.1 mM prolonged the action potential duration at 10, 50, and 90% of repolarisation. The lengthening of the action potential duration may be attributed to the substantial blockade of the outward K^+ currents caused by 1.1 mM of R(+)-pulegone (90.5% at 60 mV). These findings suggest that R(+)-pulegone exerts its negative inotropic effect on mammalian heart mainly by decreasing the L-type Ca^{2+} current and the global intracellular Ca^{2+} transient.

1. Introduction

R(+)-pulegone ((R)-2-isopropylidine-5-methyl-cyclohexanone) is a ketone monoterpene found in several plant essential oils, including those from the *Mentha* genus, such as *Mentha pulegium* (also known as pennyroyal). Pennyroyal oil, which contains between 62% and 95% R(+)-pulegone (Gordon et al., 1982; Grundschober, 1979), is traditionally used not only therapeutically but also for its toxic effects as abortifacient (Bowen and Cubbin, 1992). A number of studies have associated pennyroyal toxicity with the presence of R(+)-pulegone (Anderson et al., 1996; Bakerink et al., 1996; Gordon et al., 1982; Molck et al., 1998; Moorthy et al., 1989; Sullivan et al., 1979). Surprisingly, despite its reported toxic effects, pennyroyal oil is largely used as flavouring agent in chewing gums, toothpaste, and candies (Petrakis et al., 2009).

The complete pharmacological characterisation of essential oils and their constituents is a very active and important area of current research. In this context, several plants that produce R(+)-pulegone as a secondary metabolite were the focus of recent pharmacological investigation. Previously, the essential oil of *Mentha piperita* (peppermint) was reported to have antispasmodic effects on rat tracheal (Souza et al., 2010) and on the guinea pig gastrointestinal smooth muscle (Hills and Aaronson, 1991). Spasmolytic action on rat ileum was reported with *Calamintha glandulosa* essential oil (Brankovic et al., 2009). Similarly, relaxant effects were also reported on uterine smooth muscle exposed to the essential oil of *M. pulegium* (Soares et al., 2005), on rat vascular smooth muscle (Guedes et al., 2004), and guinea-pig intestinal smooth muscle also exposed to the essential oil of *Mentha* x *villosa* (Sousa et al., 1997). According to these reports, R(+)-pulegone likely inhibits spontaneous and potassium-induced muscle contractions, and this effect has been reported to be related

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to a significant reduction in calcium influx (Brankovic et al., 2009). However, it is important to mention that, so far, there is no direct evidence to support this mechanism of action.

Cardiac contractility is dependent on intracellular Ca^{2+} homeostasis. Such homeostasis contributes to control the action potential waveform. There is evidence that R(+)-pulegone may act through diminution of Ca^{2+} influx, which could evoke changes in cardiac contractility. This evidence led us to ask the following questions: 1) Does R(+)-pulegone directly inhibit L-type Ca^{2+} channels? 2) Does R(+)-pulegone change the cardiac intracellular global Ca^{2+} transient? 3) Does R(+)-pulegone modify cardiac action potential properties?

2. Materials and methods

2.1. Drugs

The following drugs were purchased from Sigma-Aldrich (St. Louis, Missouri, USA): R(+)-pulegone; nifedipine; reserpine; S-(−)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester (S-(−)-BAY K8644); protease type XXIII (Catalogue # P4032); porcine pancreas trypsin (Catalogue # T0303); insulin; bovine serum albumin (Catalogue # A9318); HEPES; EGTA; tetraethylammonium; CsCl; CsOH; Dulbecco's modified Eagle's medium (DMEM); and penicillin/streptomycin (Catalogue # P4333). Fluo4-AM was purchased from Molecular Probes, Inc. (Eugene, Oregon, USA). Collagenase type II was purchased from Worthington Biochemical Co. (Freehold, NJ, USA). Dimethyl sulfoxide (DMSO), NaCl, KCl, $MgCl_2$, $NaHCO_3$, $CaCl_2$, NaH_2PO_4, NaOH, glucose, and heparin were acquired from Vetec (Rio de Janeiro, Brazil) or Merck (Darmstadt, Germany).

2.2. Animals

The experiments designed to evaluate changes in contractility elicited by R(+)-pulegone were performed using guinea pigs of both sexes (*Cavia porcellus*, 400 to 700 g). Experiments on the effects of R(+)-pulegone on the L-type Ca^{2+} current, intracellular Ca^{2+} transient, action potential and potassium currents were performed on isolated ventricular cardiac myocytes from C57Bl/6J mice (both sexes). This investigation was approved by the Animal Research Ethics Committee (Protocol No. 37/10) of the Federal University of Sergipe, Brazil.

2.3. Inotropic effect of R(+)-pulegone in guinea pig left atria

The animals were sacrificed by decapitation. Each experiment was carried out on isolated guinea pig left atrium mounted in an organ chamber. There, the atrium was bathed in Tyrode's solution modified according to Dorigo et al. (1990) (in mM: NaCl 120, KCl 2.7, $MgCl_2$ 0.9, $NaHCO_3$ 11.9, $CaCl_2$ 1.37, glucose 5.5, NaH_2PO_4 0.4, pH 7.2), maintained at 29 ± 0.1 °C, and oxygenated with carbogen (95% O_2 and 5% CO_2). The atria were stretched to 5 mN and submitted to field stimulation (1 Hz). The stimulation pulses (400 V, 0.5 ms) were provided by a Digitimer 3072 Stimulator controlled by a Digitimer D4030 Programmer (Welwyn Garden City, Hertfordshire, AL7 3BE, England). Atrial contraction force was recorded with an isometric force transducer (HP FTA 10-1 Sunborn, HP 8805B, Chicago, Illinois, USA). The signals were stored in a computer to be processed and analysed off-line (DATAQ DI400, DI 205, WINDAQ PRO Acquisition, Ohio, Akron, USA).

We then determined the contractile force and times required at 50% of the maximal force amplitude for contraction (CT_{50}) and relaxation (RT_{50}). These data were measured under control conditions and after addition of R(+)-pulegone or nifedipine to the organ bath. To measure these parameters, 50 consecutive contractions were selected and processed by the software CONEXON, which was developed in the Cardiobiophysics Research Laboratory by Dr. E.A. Conde-Garcia (Patent deposit #00051104).

2.4. Effects of R(+)-pulegone on Ca^{2+} influx in guinea pig left atria

The experiments were carried out on isolated atria obtained from reserpinised guinea pigs (5 mg/kg, i.p., 24 h before the experiment). Concentration–response curves for $CaCl_2$ and BAY K8644 were obtained before and after incubating the preparation with 3.2 mM R(+)-pulegone for 15 min. The results are expressed as percentages of the maximal atrial contractile response to $CaCl_2$ or BAY K8644.

2.5. Effects of R(+)-pulegone on the L-type Ca^{2+} current, action potential and outward potassium current

Ventricular cardiomyocytes from mice (C57Bl/6J) were enzymatically isolated as previously described (Shioya, 2007), with few modifications. Whole-cell voltage-clamp recordings were obtained at room temperature (22–25 °C) using an EPC-9.2 patch-clamp amplifier (HEKA Electronics, Rheinland-Pfalz, Germany) as routinely used in our laboratory (Lara et al., 2010; Oliveira et al., 2007). After the whole-cell configuration was complete, the pipette solution was allowed to equilibrate with the intracellular environment for 3 to 5 min. The recording electrodes had tip resistances of 0.5 to 1.5 MΩ. Current recordings were low-pass filtered (2.9 kHz) and digitised at 10 kHz before being stored on a computer. Myocytes showing a series resistance (Rs) larger than 6 MΩ were not used in the analysis. Rs compensation was set at 40 to 50%. For measurements of L-type Ca^{2+} current ($I_{Ca,L}$), recording pipettes were filled with an internal solution containing (in mM) 120 CsCl, 20 TEACl, 5 NaCl, 10 HEPES, and 5 EGTA, and the pH was set to 7.2 with CsOH. $I_{Ca,L}$ was recorded in the presence of 1.8 mM extracellular Ca^{2+}. To measure $I_{Ca,L}$ steady-state inactivation, cardiac cells were perfused with an external solution containing (in mM) 135 TEACl, 10 HEPES, 20 glucose, 5.4 CsCl, 1 $MgCl_2$, and 1.8 $CaCl_2$, and the pH was set to 7.4 with CsOH.

For the time course analysis of the effects of 0.11 mM and 1.1 mM R(+)-pulegone on the L-type Ca^{2+} channels, $I_{Ca,L}$ was elicited every 10 s by test pulses from a holding potential of −80 mV to −40 mV for 50 ms to inactivate Na^+ and T-type Ca^{2+} channels. Then we stepped the membrane potential to 0 mV for 300 ms. The same protocol was performed before, during, and after washing off the R(+)-pulegone. For the current vs. voltage relationship analysis, $I_{Ca,L}$ was elicited by 300 ms test potentials ranging from −50 to 50 mV in 10 mV increments. The steady-state activation curves were obtained with the Boltzmann equation in the form: $G/G_{max} = 1/\{1 + \exp[(V - V_{0.5})/k]\}$, where $V_{0.5}$ is the voltage at which 50% of the maximum conductance was attained and k is the slope factor. The voltage dependence of $I_{Ca,L}$ steady-state inactivation was investigated using a double-pulse protocol with a 1 s conditioning voltage step to potentials between −110 and 10 mV in 10 mV increments. This was followed by a 100 ms test pulse to 0 mV to evaluate the steady-state inactivation of L-type Ca^{2+} channels. For steady-state inactivation, the peak current value at the corresponding conditioning membrane potential was normalised to the I_{max} measured at −110 mV conditioning pulse and was fitted using the Boltzmann equation: $I/I_{max} = 1/\{1 + \exp[(V - V_{0.5})/k]\}$, where $V_{0.5}$ is the voltage at which 50% of the maximum conductance was attained and k is the slope factor.

To measure action potential (AP) parameters and K^+ currents, the pipette solution was filled with (in mM) 130 K-aspartate, 20 KCl, 10 HEPES, 2 $MgCl_2$, 5 NaCl, and 5 EGTA, and the pH was set to 7.2 with KOH. We used Tyrode's solution as the bath solution, which contained (in mM) 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 10 glucose (pH set at 7.4). For the time course analysis of 1.1 mM R(+)-pulegone effects, we recorded 20–30 APs before applying the drug. We evaluated the overshoot amplitude, maximal rate of depolarisation, duration at 10, 50, and 90% of AP repolarisation,