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Pharmacological evaluation of R(+)-pulegone on cardiac excitability: Role of potassium current blockage and control of action potential waveform

Artur Santos-Miranda[b], Antonio Nei Gondim[b,c], Jose Evaldo Rodrigues Menezes-Filho[d], Carla Marina Lins Vasconcelos[d], Jader Santos Cruz[b,1], Danilo Roman-Campos[a,*,1]

[a] Departamento de Biofísica, Universidade Federal de São Paulo/Escola Paulista de Medicina, São Paulo, Brazil
[b] Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Minas Gerais, Brazil
[c] Laboratório Laboratório de Biofísica e Farmacologia do Coração, Departamento de Educação – Campus XII, Universidade do Estado da Bahia, Guanambi, Bahia, Brazil
[d] Laboratório de Biofísica do Coração, Departamento de Fisiologia, Universidade Federal de Sergipe, Aracaju, Sergipe, Brazil

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ABSTRACT

Introduction: R(+)-pulegone is a ketone monoterpene and it is the main constituent of essential oils in several plants. Previous studies provided some evidence that R(+)-pulegone may act on isolated cardiac myocytes. In this study, we evaluated in extended detail, the pharmacological effects of R(+)-pulegone on cardiac tissue.
Methods: Using *in vivo* measurements of rat cardiac electrocardiogram (ECG) and patch-clamp technique in isolated myocytes we determinate the influence of R(+)-pulegone on cardiac excitability.
Results: R(+)-pulegone delayed action potential repolarization (APR) in a concentration-dependent manner (EC_{50} = 775.7 ± 1.48, 325.0 ± 1.30, 469.3 ± 1.91 μM at 10, 50 and 90% of APR respectively). In line with prolongation of APR R(+)-pulegone, in a concentration-dependent manner, blocked distinct potassium current components (transient outward potassium current (I_{to}), rapid delayed rectifier potassium current (I_{Kr}), inactivating steady state potassium current (I_{ss}) and inward rectifier potassium current (I_{K1})) (EC_{50} = 1441 ± 1.04; 605.0 ± 1.22, 818.7 ± 1.22; 1753 ± 1.09 μM for I_{to}, I_{Kr}, I_{ss} and I_{K1}, respectively). The inhibition occurred in a fast and reversible way, without changing the steady-state activation curve, but instead shifting to the left the steady-state inactivation curve ($V_{1/2}$ from −56.92 ± 0.35 to −67.52 ± 0.19 mV). *In vivo* infusion of 100 mg/kg R(+)-pulegone prolonged the QTc (∼40%) and PR (∼62%) interval along with reducing the heart rate by ∼26%.
Conclusion: Taken together, R(+)-pulegone prolongs the APR by inhibiting several cardiomyocyte K^+ current components in a concentration-dependent manner. This occurs through a direct block by R(+)-pulegone of the channel pore, followed by a left shift on the steady state inactivation curve. Finally, R(+)-pulegone induced changes in some aspects of the ECG profile, which are in agreement with its effects on potassium channels of isolated cardiomyocytes.

Introduction

Pharmacological characterization of essential oils and/or its constituents is becoming an area of vital interest mainly to delineate their potential toxic and therapeutic effects. R(+)-pulegone ((R)-2-isopropylidine-5-methyl-cyclohexanone) (Fig. 1) is a ketone monoterpene and constitutes more than eighty percent of the essential oil of *Mentha pulegium* (also known as pennyroyal) (Gordon et al. 1982). The latter was reported to have relaxant effects on rat vascular smooth muscle (Guedes et al. 2004), isolated myometrium (Soares et al. 2005), isolated trachea and urinary bladder (Soares et al. 2012). Also organic extracts of *Mentha pulegium* have antispasmodic activity on rat ileum strips (Estrada-Soto et al. 2010). Despite the wide range of substances that comprise essential oils, usually only from one to three of their constituents account for between 20 and 70% of all them. These substances often determine

* Corresponding author. Tel.: +55 11 5576 4848 branch 2350.
E-mail addresses: drcbio@gmail.com, drcampos@unifesp.br (D. Roman-Campos).
[1] These authors contributed equally to this work.

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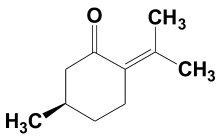

Fig. 1. Structure of (R)-(+)-pulegone.

the general biological activity of the essential oils they comprise (Ipek et al. 2005). Indeed, many studies have identified hepatotoxic effects in R(+)-pulegone containing essential oils (Anderson et al. 1996; Mizutani et al. 1987a,b; Thomassen et al. 1988).

Recently, the relaxant effects of R(+)-pulegone have been attributed to changes in membrane ion fluxes (Estrada-Soto et al. 2010; Soares et al. 2012). In fact, it was verified that R(+)-pulegone causes negative inotropism in mammalian myocardium that was related to blockage of L-type Ca^{2+} current (de Cerqueira et al. 2011), which led to impaired cardiomyocyte mechanical function. The proper control of electrical properties of heart cells is of paramount important to their function. Thus, disturbing such equilibrium usually has a deleterious impact on cardiac physiology (Bassani et al. 2004; Bers 2008). Additionally, modulation of electrical activity of cardiac myocyte by natural occurring chemical compounds may present a therapeutic application, especially in the context of cardiac arrhythmias (Li et al. 2008). Furthermore, a more comprehensive pharmacological characterization of their effects may provide additional research tools to investigate ion channel function (de Araujo et al. 2011).

Despite the previous study conducted by (de Cerqueira et al. 2011) showing that R(+) pulegone impairs the cardiac contractility by reducing Ca^{2+} and K^+ currents, so far there is no information about the effect of this drug on distinct K^+ channel subtypes expressed in cardiac myocytes. Additionally, it is not know the implications of those cellular effects on the *in vivo* cardiac electrophysiology profile. Here we describe and quantify the effects of R(+)-pulegone on the action potential (AP) parameters and on distinct K^+ current subtypes that contribute to the AP waveform in isolated cardiac myocytes. Furthermore, we investigated if these alterations can account for changes on the *in vivo* electrocardiographic (ECG) profile of rat heart.

Materials and methods

Chemicals and reagents

R(+)-Pulegone, protease type XXIII, porcine pancreas insulin, bovine serum), $CdCl_2$, TEA-Cl, NMDG, CsCl, NaCl, KCl, HEPES; EGTA, and K-aspartate were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Dimethyl sulphoxide (DMSO), $MgCl_2$, $NaHCO_3$, $CaCl_2$, NaH_2PO_4, NaOH, and D-glucose, were bought from Vetec (Rio de Janeiro, Brazil) or Merck (Darmstadt Germany). Collagenase type II was acquired from Worthington Biochemical Co. (Freehold, NJ, USA). Ketamine Chloridrate and (Cetamin®) and Xilazina Chloridrate (Xilazin were bought from Syntec (São Paulo, Brazil). Heparin was a gift from Cristália Laboratórios Farmacêuticos.

Animals

Adults (250–350 g) Wistar rats from both sex were used. The animals were maintained in a temperature controlled room on a 12-h light-dark cycle, with free access to food and water. All the experiments were performance according to Animal Research ethics committee of Federal University of Minas Gerais, Brazil and the European community guidelines.

Myocyte isolation

Adult ventricular myocytes were enzymatically isolated as described by (Shioya 2007). Myocytes were freshly isolated and stored in Tyrode' solution until they were used for experiments (within 6–8 h). Only calcium-tolerant, quiescent, rod-shaped myocytes showing clear cross striations were studied.

Patch clamp experiments

Whole-cell recordings were obtained using an EPC-9.2 patch-clamp amplifier (HEKA Electronics, Rheinland-Pfalz, Germany), at room temperature (23–25 °C). After the establishment of the whole-cell configuration, the cells were maintained for 3–5 min at rest to allow equilibration between the pipette solution and the intracellular medium. Current records were filtered at 2.9 kHz and digitally sampled at 2–10 kHz. Patch pipettes had tip resistances between 1.0 and 2.0 MΩ, and myocytes presenting series resistance above 8.0 MΩ were not used in the analysis. During recording of the action potential (AP) and K^+ currents, cardiomyocytes were maintained in a Tyrode solution, containing (in mM) 140 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 0.33 NaH_2PO_4, 1.8 $CaCl_2$, 5 HEPES and 11 glucose, pH set at 7.4 with NaOH. Pipettes were filled with an internal solution containing (in mM) 130 K-aspartate, 20 KCl, 5 NaCl, 2 $MgCl_2$, 10 HEPES, and 5 EGTA, pH set at 7.2 with KOH.

To investigate the effects of R(+)-pulegone on the action potential, the holding potential was set to −80 mV. APs were elicited by short pulses (3–6 ms) of 1 nA current at 1 Hz frequency. After 50 stable control pulses, R(+)-pulegone (from 0.01 to 3 mM) was perfused for 3 minutes, before washing out the drug. The dose-response curve was fitted according to the following equation:

$$y = A1\frac{A2 - A1}{1 + 10^{-(\log IC50 - x)p}} \quad (1)$$

where, $A1$ and $A2$ are the bottom and top asymptotes, $\log IC50$ is the 'x' drug concentration which half of the biological effect was observed, and p is the Hill coefficient.

To investigate the effect of R(+)-pulegone on cardiac potassium currents it was applied a biphasic pulse, from a holding potential of −80 to −140 mV (for 2 s) following a depolarizing pulse to +50 mV (for 4 s), every 15 s. This protocol was used to investigate the effect of R(+)-pulegone on the inward and outward components of potassium current. Myocytes were perfused with a modified Tyrode solution, in which the NaCl was replaced by N-metyl-D-glucamine (NMDG) (to abolish sodium current), and 100 μM $CdCl_2$ (to block L-type calcium current) was added. After the steady-state was achieved, R(+)-pulegone (between 0.01 and 3 mM) was applied until its steady state effect at a given concentration was achieved, followed by washout. The relative effect of each concentration was normalized and fitted by Eq. (1). The distinct subtypes of outward potassium currents were kinetically isolated: the transient outward K current (I_{to}, measured at the peak of the depolarizing pulse), delayed rectifier K current (I_{Kr}/I_{Ks}, measured 500 ms after the peak of the depolarizing pulse), and the steady state non inactivating K current (I_{ss}, measured at the end of the depolarizing pulse) (Xu et al. 1999). To investigate the action curve of the potassium currents, cells were stepped from −120 mV to +70 mV (for 3 s) from a holding potential of −70 mV in steps of 10 mV, every 15 s. The protocol was followed before, during and after washing out the 1.1 mM R(+)-pulegone. Data points were fitted according to Eq. (2).

$$I_{(V)} = G_{max} \times \frac{V_m - E_i}{1 + \exp^{(V_m - V_{0.5})}/s} \quad (2)$$

where G_{max} is the maximal conductance; V_m is the test membrane potential. E_i is the electrochemical equilibrium potential for the