Effects of eugenol on resting tension of rat atria

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Abstract

In cardiac and skeletal muscle, eugenol (μM range) blocks excitation-contraction coupling. In skeletal muscle, however, larger doses of eugenol (mM range) induce calcium release from the sarcoplasmic reticulum. The effects of eugenol are therefore dependent on its concentration. In this study, we evaluated the effects of eugenol on the contractility of isolated, quiescent atrial trabeculae from male Wistar rats (250-300 g; n = 131) and measured atrial ATP content. Eugenol (1, 3, 5, 7, and 10 mM) increased resting tension in a dose-dependent manner. Ryanodine [100 μM; a specific ryanodine receptor (RyR) blocker] and procaine (30 mM; a nonspecific RyR blocker) did not block the increased resting tension induced by eugenol regardless of whether extracellular calcium was present. The myosin-specific inhibitor 2,3-butanedione monoxime (BDM), however, reversed the increase in resting tension induced by eugenol. In Triton-skinned atrial trabeculae, in which all membranes were solubilized, eugenol did not change resting tension, maximum force produced, or the force vs pCa relationship (pCa = $-$log $[Ca^{2+}]$). Given that eugenol reduced ATP concentration, the increase in resting tension observed in this study may have resulted from cooperative activation of cardiac thin filaments by strongly attached cross-bridges (rigor state).

Key words: Eugenol; Atrial trabeculae; Resting tension; Ryanodine; Rat; ATP

Introduction

In cardiac muscle, excitation-contraction coupling (ECC) is mediated by Ca^{2+} influx through voltage-gated Ca^{2+} channels (dihydropyridine receptors, DHPRs). This limited Ca^{2+} entry triggers a massive Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR), a process that leads to activation of contractile proteins and cardiac muscle contraction (1). It is well established that CICR is mediated by the ryanodine receptors (RyRs) of Ca^{2+} release channels on the membrane of the SR (2,3). Eugenol [2-methoxy-4-(2-propenyl)phenol], an essential oil extracted from croton-clover and the chief component of clove oil, is frequently used in the food industry, in aromatherapy, and as a therapeutic agent in dentistry (4-7).

Experimental studies have demonstrated that eugenol has a broad range of biological effects. For example, at low concentrations (0.01-0.5 mM), eugenol reduces the force produced by electrically paced intact papillary muscles in rat and guinea pig model systems (8,9). The authors suggest that this effect is likely due to blockage of the L-type calcium channel.

This hypothesis is supported by a study that used the whole-cell configuration patch clamp technique on the L-type Ca^{2+} current in isolated canine and human ventricular cardiomyocytes (10). Furthermore, in skeletal muscle of toads, eugenol (at low concentrations) blocked contraction induced by a high-potassium Ringer solution, but potentiated the effect of caffeine (an RyR agonist) (11).

In the same study, however, high concentrations of eugenol (mM range) induced muscle contraction per se. Skeletal muscle contraction induced by eugenol likely involves calcium release from the SR through RyR and a heparin-sensitive pathway (12). This suggests that the effects of eugenol are dependent on the concentration used. However, in cardiac muscle, there have been no studies using eugenol in the millimolar range as has been conducted in skeletal muscle fibers. Thus, we evaluated the effects of eugenol on the contractility of isolated atrial trabeculae from the rat and measured the concentration of ATP. We found that eugenol increased the resting tension in a dose-dependent manner.

Ryanodine [100 μM; a specific ryanodine receptor (RyR) blocker] and procaine (30 mM; a nonspecific RyR blocker) did not block the increase in resting tension induced by eugenol regardless of whether extracellular

calcium was present. In Triton-skinned trabeculae, in which all membranes were solubilized, eugenol did not change the resting tension, the maximum force produced, or the force *vs* pCa relationship (pCa = −log [Ca^{2+}]). However, eugenol decreased ATP content in atrial muscle.

Material and Methods

All procedures and techniques used in this study were conducted following the guidelines of the Declaration of Helsinki for the care and use of laboratory animals, and were approved by the Animal Experimentation Ethics Committee of the Setor de Ciências Biológicas, Universidade Federal do Paraná (CEEA, #231, process #23075-021110/2007). Male Wistar rats (250-300 g; n = 131) were anesthetized with 45 mg/kg sodium pento-barbital intraperitoneally (*ip*). Hearts were removed immediately following thoracotomy and perfused with normal Ringer solution through the aortic stump to permit proper selection and dissection of the atrial muscles. The muscle ends were mounted between two forceps. One end was connected to an isometric force transducer (Scientific Instruments, GMBH, Germany) and the other end was connected to a micromanipulator. Trabeculae dimensions were measured using a reticule and a dissecting microscope. The output of the force transducer was fed into a strip-chart recorder (model RB 1020, Equipamentos Científicos do Brasil, Brazil) and a data acquisition system (Power Lab, USA) coupled to a computer for graphical analysis. Muscles were then immersed in 15-mL Ringer solution that was maintained at $30 \pm 1°C$ and gassed with 100% oxygen (see below). Muscles stretched to L_{max} (muscle length at which active tension is maximal) were stimulated by isolated rectangular pulses (10-15 V, 12 ms duration) through a pair of platinum electrodes placed along the entire extended length of the muscle. The standard stimulation rate was 0.5 Hz. Recording started after 60 min to permit the beating preparation to adapt to the new environmental conditions. In normal Ringer solution, the twitch was used to test the efficacy of the drugs used (procaine or ryanodine). After the tension reached steady state, electrical stimulation was stopped and 5 mM eugenol, with or without 100 μM ryanodine, and/or 10 mM procaine, was added to the solution. In another series of experiments, the drugs were added to calcium-free Ringer solution.

The bathing solution was normal Ringer solution, which included 110 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Trizma, and 11 mM glucose. The pH was adjusted to 7.4. Calcium was omitted to obtain calcium-free Ringer solution. Stock solutions of 10 mM ryanodine (dissolved in ethanol) and 100 mM eugenol (dissolved in distilled water) were added directly to the Ringer solution to obtain final concentrations of 100 μM

and 5 mM, respectively. Procaine and BDM (2,3-butane-dione monoxime) were prepared and used on the same day of the experiments.

Triton-skinned fibers experiments

In protocols using Triton-skinned atrial trabeculae, preparations were obtained and mounted in the same apparatus described above. The experiments were done at 28°C. The solutions used in these experiments were formulated using a computer program (written in Turbo Pascal language by Prof. Robert E. Godt, Medical College of Georgia) that described the multiple binding equilibrium of ions in solution, using previously given binding constants (13). The solutions contained 1 mM Mg^{2+}, 2 mM MgATP, 15 mM NaCP (Na_2 phosphocreatine), 5 mM EGTA, 50 mM BES [N,N-BIS (2-hydroxyethyl)-2-aminoethanesulfonic acid], 200 mM ionic strength [adjusted with potassium methanosulfonate ($KmeSO_3$)], and had a pH of 7.0. All solutions contained creatine kinase at a final concentration of 1 mg/mL. The pCa (pCa = −log [$Ca^{2+}]_o$) ranged from 8.5 and greater (relaxing solution) to 4.0 and was adjusted by appropriate addition of $CaCl_2$ and removal of $KmeSO_3$ to maintain an ionic strength at 200 mM. The force *vs* pCa relationship was determined by exposing the trabeculae to this range of pCa. These procedures were conducted in the presence and absence of eugenol to test the effects of the compound on Ca^{2+} sensitivity of the contractile machinery and maximal force (F_{max}).

Experimental solutions were added to one of a series of 3-mL capacity troughs that were milled into a Plexiglas base and stirred continuously. This allowed the transfer of the trabeculae preparations to different troughs in order to expose them to solutions containing a fixed amount of calcium. After mounting, the preparations were transferred to a relaxing solution (pCa >8.5) and stretched to 120% of the slack length (zero resting tension). The trabeculae were transferred and incubated for 20 min in relaxing solution containing Triton X-100 (0.5%, v/v). Following the skinning procedure, the trabeculae were transferred back into the relaxing solution. After F_{max} had been achieved by exposing the preparation to pCa 4.0, relaxation was obtained by transferring the preparation to the relaxing solution. The force values obtained at each pCa were normalized to F_{max}. Force-Ca^{2+} data were fitted to a Hill equation of the following form: %F_{max} = 100 [$Ca^{2+}]^N/(K^N + [Ca^{2+}]^N)$, using a nonlinear least-squares technique. N is a constant related to the steepness of the relationship and K is the calcium concentration required for half-maximal activation.

ATP content of atria muscle

In another set of experiments, both atria were dissected in Ringer solution. One atrium was transferred to Ringer solution without eugenol and the other to Ringer solution with eugenol. The preparations were kept in
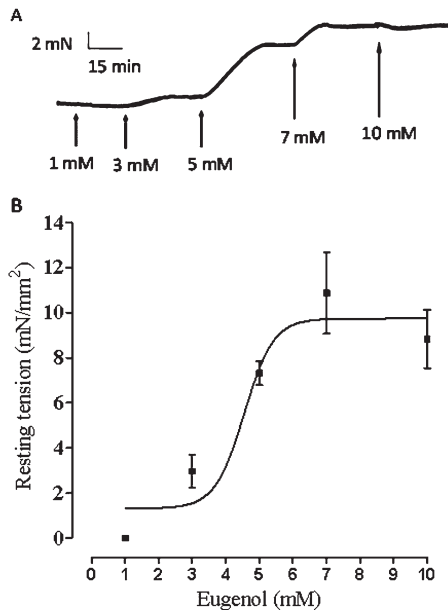
Figure 1. Effect of eugenol on the resting tension of rat atria muscle. *A*, Typical record of the effect of eugenol at different concentrations (1, 3, 5, 7, and 10 mM). *B*, Dose-response relationship of eugenol on the resting tension (n = 6).

these solutions for a few seconds (time 0), 30, or 60 min. The preparations were frozen in liquid nitrogen and then weighed. The tissues were suspended in 1000 µL 0.4 M perchloric acid and homogenized. The suspension was centrifuged for 5 min at 3000 *g* at 4°C. The perchlorate anion was precipitated by addition of 250 µL 1.0 M K_2HPO_4 to the supernatant and then removed by centrifugation for 5 min at 3000 *g* at 4°C. The supernatant was used for ATP measurements using the luciferase enzyme method. To determine luciferase activity, 20 µL of each sample was pipetted into a 5-mL polystyrene Falcon

tube and read on a luminometer (GloMax-Promega, USA) using the Promega luciferase assay substrate. Tests were done in duplicate. Protein concentrations in 20 µL of sample were determined using a BioRad assay (USA). Data were normalized by protein concentration.

Reagents

Eugenol (purity = 99%), ryanodine, BDM, procaine, MgATP, disodium phosphocreatine, EGTA, imidazole, methanesulfonic acid, calcium chloride, and creatine kinase were purchased from Sigma Chemical Co. (USA). All other reagents were analytical grade and were purchased from Merck (Germany).

Statistical analysis

Results are reported as means ± SE of at least 6 observations. For multiple comparisons, statistical evaluations were performed using ANOVA followed by the Student-Newman-Keuls test for paired multiple comparisons. Results were considered significantly different from controls when P<0.05.

Results

Intact atrial trabeculae

The effect of eugenol on intact atrial trabeculae is reported in Figure 1. Eugenol induced an increase in the resting tension in a dose-dependent manner with an EC_{50} of approximately 5 mM.

Twitch was blocked (80-100%) by the presence of procaine, ryanodine, or BDM in all electrically stimulated preparations, demonstrating the efficiency of the drugs (results not shown). After 1-2 min exposure to calcium-free Ringer solution, twitch was also abolished. No alteration in resting tension was observed under any of these conditions. The increase in the resting tension induced by eugenol was observed in both normal and calcium-free Ringer solution, as shown in Figures 2, 3,
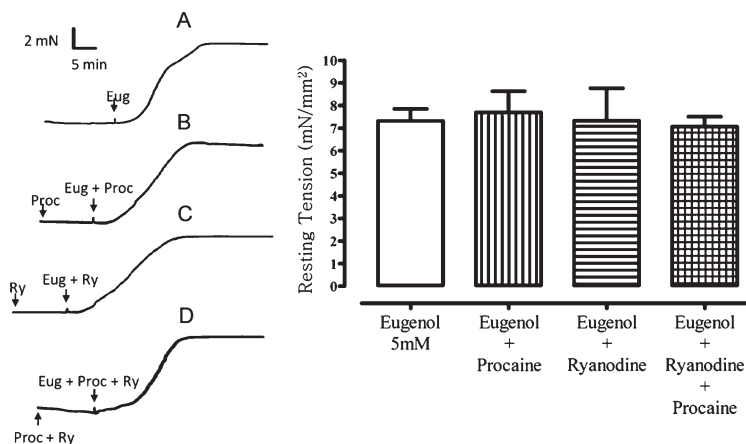

Figure 2. Effect of eugenol (Eug) on the resting tension of atria trabeculae of the rat in the absence (*A*) and in the presence of procaine (Proc) (*B*), ryanodine (Ry) (*C*) or both, procaine and ryanodine (*D*). The experiments were carried out in normal Ringer solution. No significant differences in force were observed among groups (P>0.05, ANOVA followed by the *t*-test; n = 6).
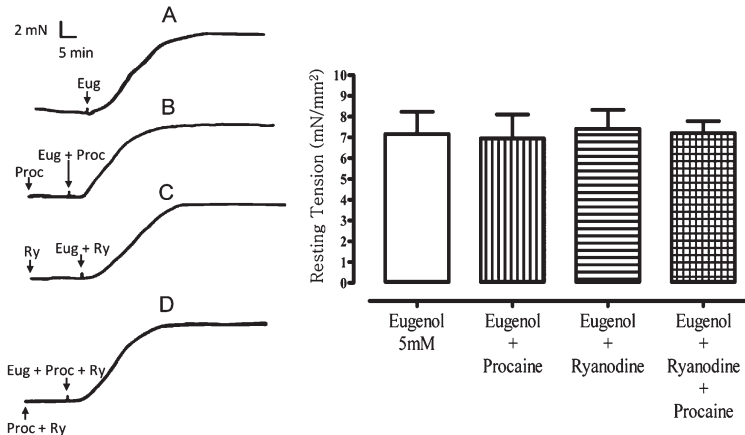
Figure 3. Effect of eugenol (Eug) on the resting tension of atria trabeculae of the rat in the absence (*A*) and in the presence of procaine (Proc) (*B*), ryanodine (Ry) (*C*) or both, procaine and ryanodine (*D*). The experiments were carried out in calcium-free Ringer solution. No significant differences in force were observed among groups (P>0.05, ANOVA followed by the *t*-test; n=6).

and 4. The magnitude of this increase was equivalent in both solutions. This effect of eugenol was also observed in preparations previously exposed to procaine and/or ryanodine, either in normal or calcium-free Ringer solutions. In normal Ringer solution containing both procaine and ryanodine, the increase in the resting tension did not differ from control values. However, after 20-30 min, the effect of eugenol on resting tension was completely reversed by exposing the preparation to BDM (Figure 4). The effect of BDM was completely reversible. However, 5-10 min after this compound had been washed out, eugenol induced an increase in resting tension. Under all experimental conditions tested, 5 mM eugenol increased the resting tension to 7.318 ± 0.529 mN/mm^2,

which corresponded to 118% of F_{max}.

Triton-skinned atrial trabeculae

All trabecular membranes were solubilized when exposed to a skinning solution containing Triton X-100; however, the contractile apparatus was kept intact and functional. In preparations skinned with Triton, eugenol was unable to induce an increase in resting tension. Furthermore, eugenol did not alter other parameters (Hill coefficient, $[Ca^{2+}]_{50}$, or F_{max}) obtained based on the force *vs* pCa relationship, as demonstrated in Figure 5. The values of N and Ca^{2+}_{50} in the absence and in the presence of eugenol were 1.37 ± 0.03 and 1.60 ± 0.2 µM, and 1.18 ± 0.02 and 1.24 ± 0.17 µM, respectively.

ATP content of atrial muscles

The concentration of ATP in atrial muscle is shown in Figure 6. Eugenol reduced the concentration of ATP in a time-dependent manner. In the absence or presence of eugenol, the ATP concentrations at 0, 30, and 60 min
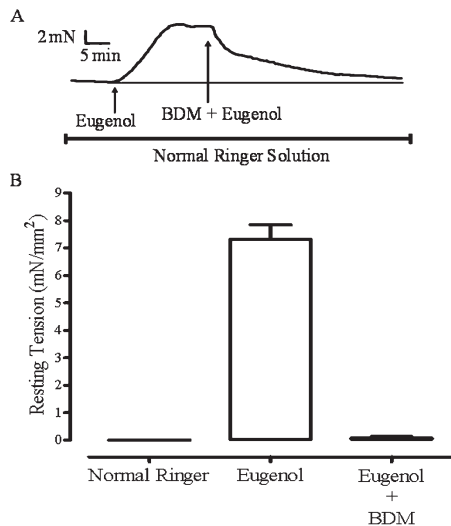

Figure 4. *A*, Typical chart record showing the effect of 30 mM 2,3-butanedione monoxime (BDM) on the contraction induced by 5 mM eugenol in rat atria. *B*, Data are reported as means ± SE of the effect of eugenol and eugenol plus BDM. A significant difference in resting tension was observed (P<0.05, eugenol *vs* eugenol+BDM, Student *t*-test; n=6).
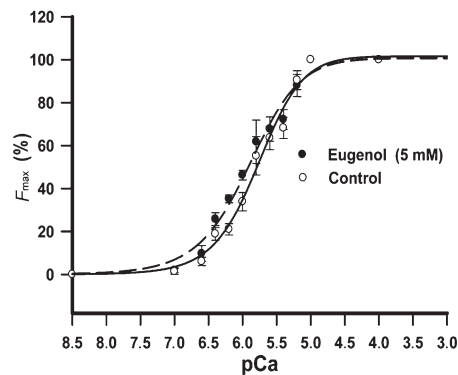

Figure 5. Force-pCa relationship of Triton-skinned atria trabeculae. Force was normalized to F_{max}. Data are reported as average force ± SE. The values of N and Ca^{2+}_{50} in the absence and in the presence of eugenol were 1.37 ± 0.03, 1.60 ± 0.2 and 1.18 ± 0.02, 1.24 ± 0.17 µM, respectively (n=6).
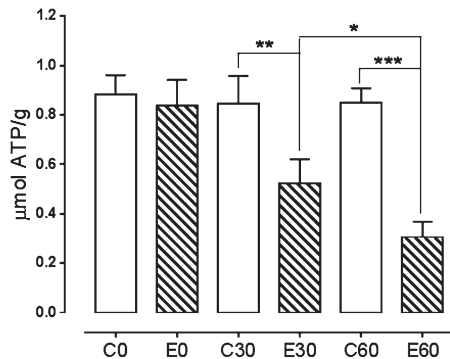
Figure 6. Effect of eugenol on ATP concentration of rat atria. The concentrations of ATP were measured in the absence (*C*) and in the presence (*E*) of eugenol at 0, 30, and 60 min. Data are reported in µmol ATP/g protein. P<0.05 (ANOVA followed by the Student *t*-test): *E30 *vs* E60; **C30 *vs* E30; ***C60 *vs* E60.

were 0.882 ± 0.036 and 0.838 ± 0.047; 0.844 ± 0.051 and 0.524 ± 0.044 (P<0.001); 0.849 ± 0.026 and 0.306 ± 0.028 µmol/g protein (P<0.0001), respectively. The ATP concentration obtained in our study was one order of magnitude lower than that reported in other studies (14, 15). The reasons for such differences are unclear but may have resulted from the use of different species, tissues sample (ventricle vs atria), or techniques. However, in our study, in the same experimental conditions, one atrium was used as a control and the other was exposed to eugenol. This procedure minimized experimental error arising from bias.

Discussion

In this study, we demonstrated that eugenol increased resting tension in a concentration-dependent manner. Because it has been demonstrated that eugenol blocks an L-type calcium channel (8), and because the increase in the resting tension was not blocked by calcium-free Ringer solution, it is unlikely that this effect was due to calcium inflow. Furthermore, the increase in resting tension likely did not involve calcium released from the SR (through RyR channels) because ryanodine and procaine did not block this effect. It appears that its effect was dependent on the proper function of the cross-bridges, as the increase of the resting tension induced by eugenol was completely inhibited by BDM, a compound that abolishes muscle contraction by decreasing the attachment rate of cross-bridges, the instantaneous number of attached cross-bridges, and the force generated per attached cross-bridge (16). The increase in resting tension may have been a consequence of the direct effect of eugenol on the contractile apparatus, i.e., increasing calcium sensitivity. However, this is unlikely since eugenol did not change resting tension or the force *vs* pCa relationship in Triton-skinned trabeculae. The Triton-skinned trabecula data also exclude the possibility of a deleterious effect of eugenol on the contractile apparatus. Thus, the increase in resting tension cannot be attributed to the extraction of troponin I from the thin filament, which may activate the contractile system. This was confirmed by the observation that eugenol did not induce an increase in resting tension after the membrane solubilization produced by Triton treatment. Because eugenol reduced atrial ATP content, the increase in resting tension observed in intact atria preparations may have been the result of a reduction in the intracellular concentration of ATP. This reduction in the ATP concentration induced by eugenol has also been demonstrated in fibroblasts (14). In isolated rat liver mitochondria, Usta et al. (15) showed that eugenol inhibited NADH oxidase, a proton pumping site, which resulted in a decline in ATP. In skeletal as well as in cardiac muscle, the reduction in the MgATP at a critical level may lead to the formation of both noncycling (rigor, ATP-free) and cycling (ATP-bound) cross-bridges by inducing cooperative activation of the thin filament (17,18). Such a mechanism was inoperative in Triton-skinned atria because the concentration of MgATP in the bathing solution was sufficiently high to avoid noncycling cross-bridge formation. In summary, we found that eugenol induced an increase in atrial resting tension through a mechanism that may have involved cooperative activation of the cardiac thin filaments by strong attached cross-bridges (rigor state).

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