

(+)-Usnic Acid Isolated from the Lichen *Cladonia substellata* Impairs Myocardial Contractility



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Supporting information Details on the chemical characterization of UA from *C. substellata* are available online at <http://www.thieme-connect.de/products>

ABSTRACT

The scientific interest in (+)-usnic acid has grown because of its antitumor, cytotoxic, and antimicrobial activities as well as weight loss. However, overuse of usnic acid has been related with severe hepatotoxicity, making its use questionable. In this study, we decided to expand the knowledge of usnic acid biological activities by characterizing its effects on the mammalian myocardium as a potential pharmacological target. Usnic acid was isolated from samples of *Cladonia substellata* and submitted to chemical characterization. Molecular inclusion complexes of usnic acid with hydroxypropyl β -cyclodextrin were prepared to improve its water solubility. The effects of usnic acid on the atrial contractility and Ca²⁺ influx were carried out in the left atrium of guinea pigs and the effect of usnic acid on the L-type Ca²⁺ current was performed in rat ventricular cardiomyocytes enzymatically isolated. To evaluate the membrane integrity of cells subjected to usnic acid, we used histological procedures. Usnic acid reduced atrial contraction with an EC₅₀ of 43.0 ± 1.0 μ M. This effect was related to a reduction of Ca²⁺ entry in myocardial cells. In isolated cardiac myocytes, usnic acid at 100 μ M inhibited the L-type Ca²⁺ current by 73.0%. In addition, usnic acid caused an irreversible myocardial contracture, reflecting a serious disturbance of the intracellular Ca²⁺ homeostasis. Such an effect could not be ascribed to tissue death because cell membrane integrity was confirmed by histological observation. Taken together, our results show that usnic acid impairs cardiac function. Clearly more studies will be necessary to allow further applications of this natural product.

Introduction

Usnic acid (UA; 2,6-diacetyl-7,9-dihydroxy-8,9b-dimethylidibenzofuran-1,3[2H,9bH]-dione) is one of the most studied lichen secondary metabolites. Several biological properties have been ascribed to this compound including antibacterial [1, 2], anti-*Trypanosoma cruzi* [3], antiviral [4], anti-inflammatory [5], apoptosis inducer [6], and antiproliferative [2, 7, 8]. UA is toxic to human lymphocytes and hepatocytes [6, 9–13]. Scientific interest in UA has grown after a number of reports showing antiproliferative effects of UA for cancerous cell lines [14–17].

However, despite beneficial effects, there are few studies demonstrating that UA alters Ca^{2+} homeostasis, induces endoplasmic reticulum (ER) stress, and increases oxidative stress, and that these alterations are involved in UA-induced cellular damage [18, 19]. Furthermore, several compounds of natural origin are Ca^{2+} channels blockers [20, 21].

UA has also been known to be a strong mitochondrial uncoupler, which could be related to its hepatotoxicity [12]. However, it has not been clarified whether UA causes toxicity in other organs such as the heart. For example, it is reported that a 14-day administration of UA (100 mg/kg/day) provoked mitochondrial swelling in cardiac myocytes in rats [22]. However, there are few studies determining whether UA has a pharmacological effect on the function of heart. Based on these results, we have attempted to determine the potential pharmacological effect of UA on heart function.

Results

The identity of usnic acid and its optical activity are reported in Supporting Information.

In the guinea pig left atrium, UA reduced the myocardium contractility in a concentration-dependent manner (► **Fig. 1a**). UA at 800 μM strongly reduced the atrial force from 29.7 ± 0.6 mgf/mg to 1.2 ± 0.5 mgf/mg (96 %, $n = 8$, $p < 0.0001$; ► **Fig. 1b**) with an EC_{50} of 43.0 ± 1.0 μM . Nifedipine, used as a positive control, presented a very similar pattern, reducing the atrial contractile force from 28.3 ± 2.1 mgf/mg to 2.5 ± 0.7 mgf/mg (91 %, $p < 0.001$, $\text{EC}_{50} = 1.1 \pm 1.0$ μM ; ► **Fig. 1b**). UA is 39-fold less potent than nifedipine. Furthermore, an increase of the atrial diastolic tension to 650.0 ± 25.97 mgf ($\text{EC}_{50} = 589.8 \pm 10.3$ μM , $n = 8$; ► **Fig. 1a,c**) was observed after incubating the atrium with 800 μM UA.

No significant histological differences between the control atria (► **Fig. 2a,c**) and the atria exposed to 800 μM UA (► **Fig. 2b,d**) were observed. Furthermore, neither muscular necrosis nor interstitial fibrosis was seen.

As demonstrated in ► **Fig. 3**, UA abolishes the Bowditch effect. ► **Fig. 3a (i and ii)** depicts representative records obtained before and after adding 70 μM UA to the bath, respectively. ► **Fig. 3a (i; control)** shows an increase in the force amplitude from 197 mgf (20 bpm) to 636 mgf (80 bpm). However, when 70 μM UA were added to the bath, the Bowditch phenomenon was completely abolished ► **Fig. 3a (ii)**. Similar results were obtained on the other three atria (► **Fig. 3b**) tested at rates from 20 bpm to 120 bpm. Note that when 70 μM UA were added to the bath solution, the atrial contractile force during the overdrive was greatly reduced to 3.33 ± 3.21 % of the control contractile force amplitude ($n = 3$,

$p < 0.01$). This result indicates that UA decreases the atrial contractile force by reducing Ca^{2+} influx in cardiac myocytes.

To further explore the idea that UA reduces Ca^{2+} influx we made use of another experimental approach. In order to examine that possibility, the extracellular Ca^{2+} concentration was changed in the absence and in the presence of UA at 100 μM . ► **Fig. 3c** illustrates the expected positive inotropic response to increases in extracellular Ca^{2+} concentration (0.5 to 5.0 mM). The calculated EC_{50} for Ca^{2+} was 0.69 ± 0.42 mM. However, when the atria were preincubated with 100 μM UA, a rightward shift of the concentration-response curve for Ca^{2+} was observed and the EC_{50} increased to 1.28 ± 0.24 mM ($p < 0.05$). These data support the conclusion that the cardiodepressor effect of UA is related to a decrease in sarcolemmal Ca^{2+} influx.

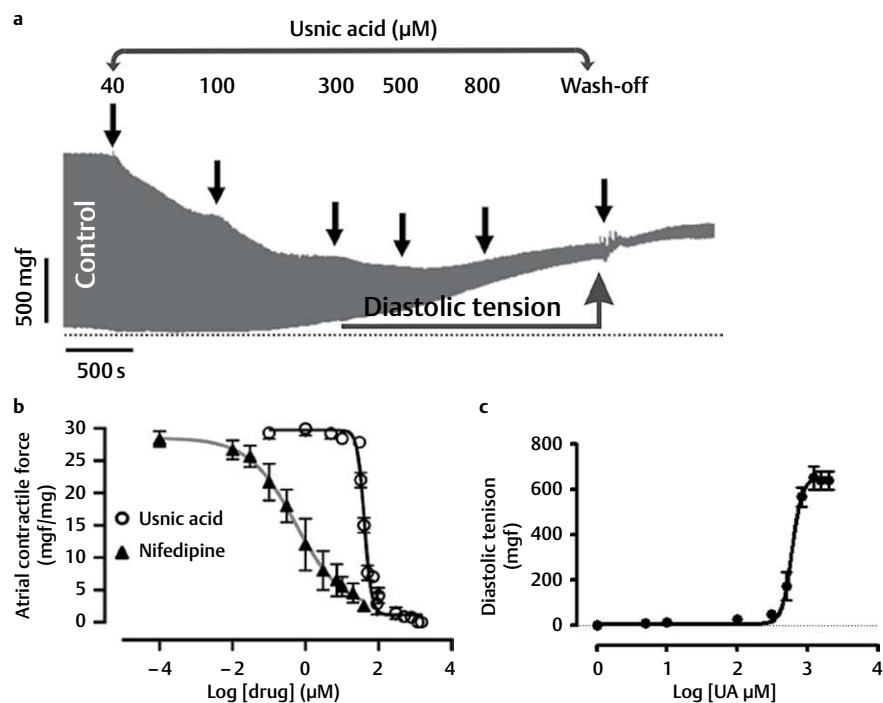
Representative families of whole-cell L-type Ca^{2+} currents are illustrated in ► **Fig. 4a** (Control) and **B** (in the presence of UA). Application of UA produced a concentration-dependent decrease of peak $I_{\text{Ca-L}}$ (mean data shown in ► **Fig. 4c**). ► **Fig. 4d** shows the effect of 1, 10, 30, and 100 μM UA on the mean I-V relationship of $I_{\text{Ca-L}}$. UA decreased the $I_{\text{Ca-L}}$ amplitude over the entire voltage range tested but did not shift the I-V relationship. The reduction of the $I_{\text{Ca-L}}$ can account for the negative inotropic effect elicited by UA in the heart.

Discussion

This study reports new findings on the effect of UA on atrial contractility as well as on the L-type Ca^{2+} current in myocardial cells. We have demonstrated that UA is a potent depressor of cardiac contractility. In addition, concentrations higher than 300 μM induced a sustained and irreversible contracture during the diastolic phase. These results cannot be explained in response to changes in the pH of the Tyrode's solution due to the use of an acidic substance (UA $\text{pK}_a = 4.4$), as the pH remained unchanged even at 1 200 μM UA (data not shown). Neither can it be attributed to cytotoxicity. There are some studies indicating a cytotoxic effect for UA at concentrations in the micromolar range [12, 17]. However, the histological analyses revealed no significant differences between the control atria and those subjected to UA (at 800 μM). Viable myocardial cells were observed showing preserved structures, including the pattern of striations that is an important parameter for cellular integrity. Therefore, UA effects on mammalian myocardium are not completely due to its claimed cytotoxicity.

This is the first report demonstrating that UA decreases cardiac contractility by blocking voltage-dependent L-type Ca^{2+} channels. Our data showed that when the atrium was incubated with UA, the Bowditch effect was critically impaired, probably due to reduction of Ca^{2+} entry into the myocardial cells. Following this line of reasoning, we performed experiments to evaluate whether positive inotropic interventions could be attenuated by UA. Our results showed that UA drastically affected positive inotropism induced by Ca^{2+} , corroborating the hypothesis that UA can reduce the sarcolemmal Ca^{2+} inward current.

In cardiac muscle, Ca^{2+} influx through the sarcolemmal membrane is essential for triggering Ca^{2+} release from the sarcoplasmic reticulum (SR). The combination of Ca^{2+} influx and SR Ca^{2+} release



► **Fig. 1** The effects of (+)-UA on guinea pig left atrium contractile force. **a** Exemplar of an original record of a negative inotropic effect elicited by (+)-UA. At concentrations above 300 µM, an increase in diastolic tension is also observed. **b** Concentration-response curves of UA ($EC_{50} = 43.0 \pm 1.0 \mu\text{M}$, $n = 8$, open circles) and nifedipine ($EC_{50} = 1.1 \pm 1.0 \mu\text{M}$, $n = 8$, open triangles). **c** Concentration-response curve of UA on diastolic tension ($EC_{50} = 589.8 \pm 10.3 \mu\text{M}$, $n = 8$, black circles).

promotes an increase in the free intracellular Ca^{2+} concentration, causing the muscle to contract [23]. The relationship between UA and the voltage-dependent L-type Ca^{2+} current was established by direct measurements of the L-type Ca^{2+} current in isolated cardiomyocytes. Our data showed that UA reduced the L-type Ca^{2+} current by 73%, which explains the negative inotropism.

We speculate that the diastolic contracture observed at high concentrations of UA may be due to an impaired Ca^{2+} reuptake from the sarcoplasm to SR lumen by sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA). We can argue that Ca^{2+} uptake is dependent on the levels of intracellular ATP, and a decrease in ATP leads to a decrease in SERCA activity and contributes to an increase in cytosolic Ca^{2+} .

These new findings offer additional mechanistic insight into how heart contractility is altered by UA and raise further questions about the safety of its medicinal use.

Materials and Methods

Lichenic material

Samples of *Cladonia substellata* Vain. (Cladoniaceae) were collected in March (fall season) in the National Park of the Itabaiana Ridge (180–670 m; $10^{\circ}41'22''\text{S}$; $37^{\circ}24'10''\text{W}$), Itabaiana, Sergipe, Brazil. They were identified by Dr. Marcelo Pinto Marcelli, Head of the Mycology and Lichenology Section of the Institute of Botany, Univer-

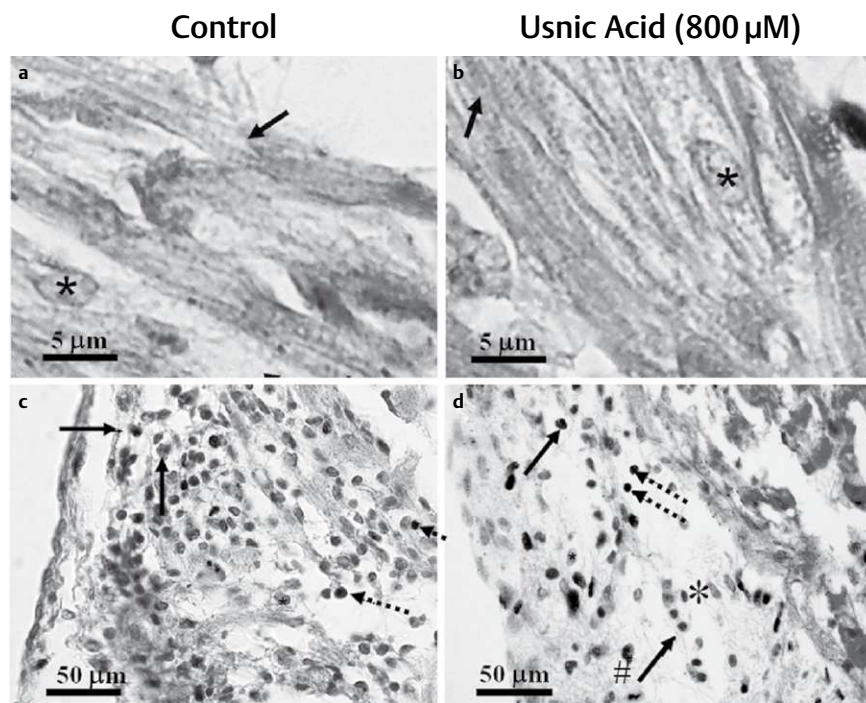
sity of São Paulo, São Paulo, Brazil, where a voucher specimen is stored (Deposit #SP393249).

Chemical characterization of usnic acid from *Cladonia substellata*

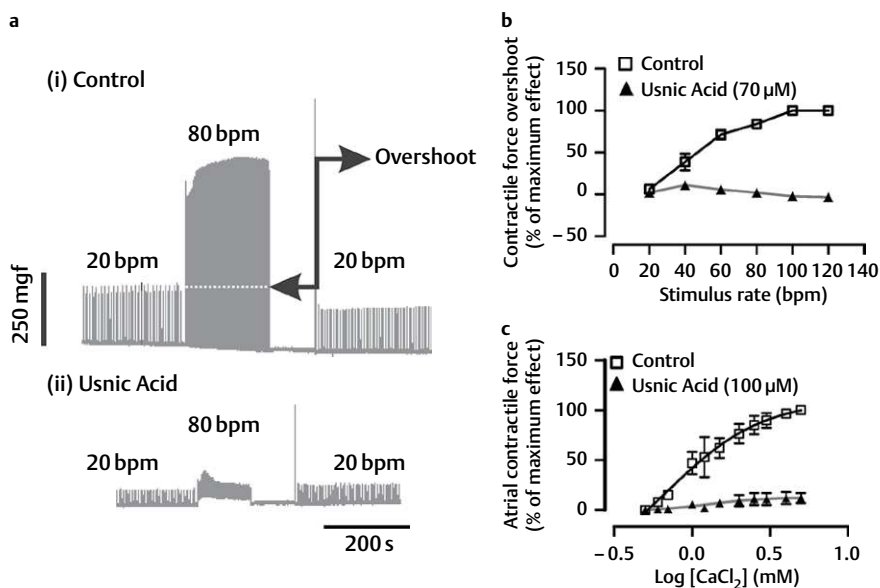
UA was chemically identified by the following procedures: 1) elemental analysis for carbon, hydrogen, and nitrogen content (Perkin-Elmer Analyzer, Model 2400), 2) NMR (Bruker DRX 500 spectrometer, ^1H : 500 MHz; ^{13}C : 125 MHz), 3) X-ray diffraction (XRD) patterns (Siemens, Model D5000, tube of $\text{CuK}\alpha$) with measurements performed from 3 to 65° (2θ) and 1 s pass time using the Powder X-ray diffraction method, 4) infrared absorption (FTIR), and 5) thermal analysis procedures [thermogravimetry (TG), differential thermogravimetry (DTG), differential scanning calorimetry (DSC)] performed according to Nunes et al. [24]. The enantiomeric form of UA isolated from *C. substellata* was determined via polarimetry (Polarimeter Jasco Model P-2000). The original spectra are available as Supporting Information.

Water solubilization of usnic acid

The water solubility of UA was improved by dissolving it in hydroxypropyl- β -cyclodextrin (HP β C) following the procedure described by Kristmundsdóttir et al. [25]. The HP β C interaction with UA was previously demonstrated [26]. Our results showed that HP β C reduced the atrial force slightly only at high concentrations (800 µM).



► **Fig. 2** (+)-UA did not affect cellular integrity. Control group (a) and 800 μM UA group (b) showing rhabdomyocytes (*) with well-defined nuclei and transverse striations (arrows). Control group (c) and 800 μM (+)-UA group (d) depicting infiltrate in the stromal cardiac connective tissue composed of lymphocytes (dotted arrows), plasma cells (full arrows), and macrophages (#) in addition to fibroblasts (*).



► **Fig. 3** Bowditch phenomenon is abolished by (+)-UA. **a** Example of atrial contractile force recordings showing the Bowditch phenomenon promoted by a sudden increase of the stimulation rate from 20 to 80 bpm (i: control and ii: in the presence of UA). **b** Average contractile force overshoot as a function of different pacing rates ($n = 3$). **c** The effects of (+)-UA on the concentration-response curves for the positive inotropic effects of CaCl_2 . At 100 μM , (+)-UA significantly reduced the positive inotropic effect of CaCl_2 ($n = 3$).

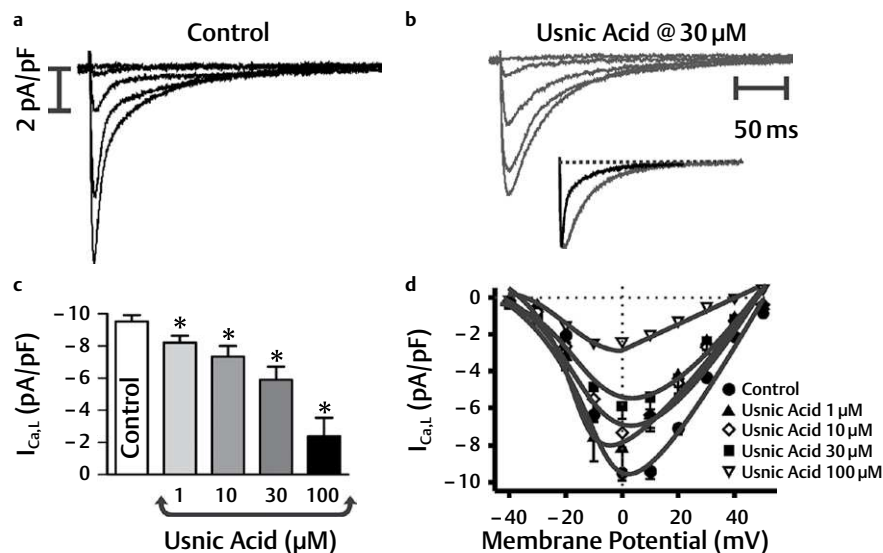


Fig. 4 (+)-UA inhibits L-type Ca²⁺ currents. Representative L-type Ca²⁺ current recordings in the absence (**a** Control) and in the presence of (+)-UA (**b** 30 μM). **c** Average L-type Ca²⁺ current density at 0 mV test pulse at 1, 10, 30, and 100 μM (+)-UA (* p < 0.05). **d** Average L-type Ca²⁺ current density vs. voltage relationships obtained at different (+)-UA concentrations. Data points are the mean ± SE.

Myocardial contractility

The effects of UA on myocardial inotropism were carried out using the left atrium of guinea pigs (*Cavia porcellus*, male, 400–500 g), which was mounted in a vertical organ bath for contractile recordings [27]. Briefly, guinea pigs were sacrificed by decapitation, the heart was quickly removed, and the left atria were mounted in an organ chamber for the contractile force recordings. The atria, under 1 gf tension and electrically stimulated (2 Hz), were immersed in oxygenated Tyrode's solution (29 ± 0.1 °C, 95 % O₂ and 5 % CO₂). The atrial contraction force was recorded with an isometric force transducer (HP FTA 10-1 Sunborn, HP 8805B). The contractile signals were amplified by an HP7754B Carrier Amplifier and then digitalized in an A/D converter (DATAQ DI400, DI 205, WINDAQ PRO Acquisition) before being stored in a computer to be processed off-line. The experimental setup is described in [28]. The following experimental procedures were performed: 1) concentration-response curve for UA, using nifedipine as the positive control, 2) effect of UA on the positive staircase (Bowditch phenomenon) elicited by a sudden increase of the stimulation rate (control rate: 12 bpm; test rate ranging from 20 bpm to 120 bpm), and 3) effect of UA on the concentration-response curve for CaCl₂ (ranging from 0.5 mM to 5.0 mM). The Bowditch phenomenon is characterized by an increase of the myocardial force occurring when the rate of stimulation is suddenly increased. It was demonstrated that the resulting contractile force enhancement is due to an increase in Ca²⁺ influx into myocardial cells [28–31].

Histological analysis

Histological procedures were used to evaluate the membrane integrity of cells subjected to a high concentration of UA (800 μM). The experiments were performed on right and left guinea pig atria

mounted separately in different organ baths. The right atrium was used as a control and the left atrium was used as the test sample (800 μM UA for 20 min). In the chambers, both atria were bathed with control Tyrode's solution (27 ± 0.1 °C) under a resting tension of 1 gf and subjected to electrical field stimulation with suprathreshold pulses (2 Hz). After that time, the atria were fixed in formaldehyde (10%, pH = 7.4), sliced, and stained with hematoxylin-eosin. Histopathological analysis was performed with an optical microscope (Olympus BX31) coupled to an edge detector.

Electrophysiological studies

Isolation of rat ventricular cardiomyocytes

To study the effect of UA on the L-type Ca²⁺ current (I_{Ca,L}), rat ventricular cardiomyocytes were enzymatically isolated [32]. Briefly, isolated hearts were subjected to aortic perfusion with a Ca²⁺-free solution (5 min) and then digested in a solution containing collagenase (Worthington, Type II) to dissociate the myocardial cells. Cardiomyocytes were then obtained and stored at room temperature. The cells were used in the first 4–6 h after isolation. Only cells that were Ca²⁺ tolerant, rod-shaped, and showed clear cross striations were used for experimentation.

Patch-clamp recordings

Whole-cell patch-clamp recordings were obtained using an EPC-9.2 patch-clamp amplifier (HEKA Electronics). After attaining the tight-seal whole-cell configuration, 3 to 5 min were allowed to equilibrate the cell interior with the pipette solution. The recording electrode resistance ranged from 1.0 to 1.5 MΩ, and experiments were carried out at room temperature (24–27 °C). Cell capacitance and series resistance were compensated before measuring the ionic current. The records were low-pass filtered (2.9 kHz)

and sampled at 10 kHz. Cardiomyocytes showing a series resistance (R_s) larger than $10\text{ M}\Omega$ were discarded. The R_s of selected cardiomyocytes was routinely compensated for by 40 to 70% before measuring the $I_{Ca,L}$. This measurement was performed using recording pipettes filled with internal solution (see composition below) and using the bath solution as the external solution (see composition below). The pulse protocol was performed as follows: first, the membrane potential was stepped from a holding potential of -80 mV to -40 mV , where it remained for 50 ms to inactivate the Na^+ channels. Then, the cell was clamped at different voltages from -40 to $+50\text{ mV}$ for 300 ms in 10 mV steps.

Solutions and reagents

Tyrode's solution (in mM): 120.0 NaCl, 2.7 KCl, 0.9 MgCl_2 , 11.9 NaHCO_3 , 1.37 CaCl_2 , 5.5 Glucose, 0.4 NaH_2PO_4 . Calcium-free solution (in mM): 130.0 NaCl, 5.4 KCl, 0.5 MgCl_2 , 0.33 NaH_2PO_4 , 3.0 pyruvate, 22.0 glucose, and 25.0 HEPES, pH 7.4 adjusted with NaOH. Digest solution: prepared by adding 1 mg/mL type II collagenase (Worthington Biochemical Co.) to the calcium-free solution. External solution (in mM): 140 NaCl, 5.4 KCl, 1 MgCl_2 , 1.8 CaCl_2 , 10 HEPES, 10 glucose, and pH set to 7.4 with NaOH. Internal pipette solution (in mM): 120.0 CsCl, 20.0 TEACl, 5.0 NaCl, 10.0 HEPES, 5.0 EGTA, pH adjusted with CsOH to 7.2. Nifedipine (purity $\geq 98\%$) was purchased from Sigma-Aldrich. Chloroform, ethanol, and reagents were of the highest grade available and were purchased from Sigma-Aldrich, Merck, J. T. Backer, Amresco, or VETEC.

Ethical committee approval

This work was previously approved by the Ethical Committee on Animal Research of the Federal University of Sergipe (CEPA/UFS, protocol #77/11, September 9, 2011). Animal handling was in compliance with the Principles of Laboratory Animal Care (NIH publication 86-23, revised 1985; <http://oacu.od.nih.gov/regs/index.htm>).

Statistical analysis

One-way ANOVA (MINITAB, Minitab Inc., EUA) followed by Tukey's post hoc test was employed to determine the significance of the difference between means. Data are reported as means \pm SE.

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Conflict of Interest

The authors declare no conflict of interest.

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