Article

Erythroxylum pungens elicits vasorelaxation by reducing intracellular calcium concentration in vascular smooth muscle cells of rats

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Abstract: The cardiovascular effects elicited by the ethanolic extract obtained from the roots of *Erythroxylum pungens* O.E. Schulz, Erythroxylaceae (EEEP) and the vasorelaxant effect induced by its main tropane alkaloid (pungencine) were investigated. In normotensive rats, administration of EEEP (1, 10, 30 and 60 mg/kg *i.v.*, randomly) produced dose-dependent hypotension (-2±1, -7±0.5 -17.6±1, -24±1 Δ mmHg, n=5) followed by tachycardia (3±0.5, 7±2, 7.1±1, 10±5 Δ bpm, n=5). In intact phenylephrine (Phe, 10 μM)-pre-contracted rings, EEEP (0.01-500 μg/mL) induced concentration-dependent vasorelaxation (EC50 13.7±5.5 μg/mL, Maximal Response= 92±2.6%), and this effect was unchanged after the removal of the vascular endothelium (EC50 27.2±4.7 μg/ml, Maximal Response= 88.3±3.3 %). In KCl (80 mM)-pre-contracted-endothelium-denuded rings, EEEP elicited concentration-dependent relaxation (EC50= 128.2±11.2 μg/mL, Maximal Response 76.8±3.4%). Vasorelaxation has also been achieved with tonic contractions evoked by the L-type Ca²⁺ channel agonist Bay K 8644 (EC50 80.2±9.1 μg/mL, Maximal Response 86.3±8.3%). In addition, in a depolarizing medium, EEEP inhibited CaCl₂ (30-500 μg/mL) induced contractions and caused a concentration-dependent rightward shift of the relaxation curves. Lastly, the tropane alkaloid pungencine caused vasorelaxation in mesenteric arteries resembling to the EEEP responses. These results suggests that EEEP induces hypotension and vasorelaxation, at least in part, due to the reduction in [Ca²⁺]i in vascular smooth muscle cells.

Introduction

Medicinal plants contain several classes of bioactive compounds such as polyphenols, alkaloids and terpenes (Oliveira et al., 1996; Barbosa-Filho et al., 2000; Adaramoye et al., 2009). Among the pharmacological activities of compounds isolated from medicinal drugs on the cardiovascular system are calcium antagonism, NO release, antioxidant and others (Guedes et al., 2004; Ribeiro et al., 2010).

The Erythroxylaceae is an important family that produces active secondary metabolites on biological systems. *Erythoxylum* species are known for the production of the tropane diester alkaloid, cocaine, but only a few members of this genus accumulate this alkaloid in quantity (Chin et al., 2006; Queiroz et al., 2009). This family has four genera, with approximately 250 species, being the genus *Erythroxylum* P. Browne, which is the largest one, found widely distributed in tropical regions of South America, Africa, Southeast Asia, and Madagascar (Plowman & Hensold, 2004; Brock et al., 2005). In South America, Brazil is one of the main centers of diversity and endemism for *Erythroxylum* sp., where around 114 are found from a total of 187 species catalogued (Brock et al., 2005). *Erythrxoxylum pungens*, popularly known as "rompe-gibão" is found in the Northeast region of Brazil.

Among their constituents with biological activity, tropane alkaloids demonstrated analgesic effects, anesthetic, anticholinergic, antiemetic, antihypertensive, parasympatholytic, and many others pharmacological actions (Grynkiewicz & Gadzikowska, 2008). In modern medicine, three tropane alkaloids, atropine, hyoscyamine, and scopolamine, are among the major drugs obtained from plants. Although synthetic approaches have been developed for the basic tropane alkaloids, most of the pharmaceutically important alkaloids of this series are still obtained from plant sources (Lounasmaa & Tamminen, 1993; Khattak et al., 2002), including *Erythroxylum pungens* (Oliveira et al., 2010).

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To our knowledge, there is no information about the effects of *Erythroxylum pugens* ethanolic extract (EEEP) or tropane alkaloid pungencine on the cardiovascular system. Therefore, the present study was designed to determine whether EEEP and its isolated tropane alkaloid (pungencine, **1**) has a pharmacological effect on isolated rat superior mesenteric arterial rings, and if so, to unravel the mechanisms by which EEEP exerts its cardiovascular effects.

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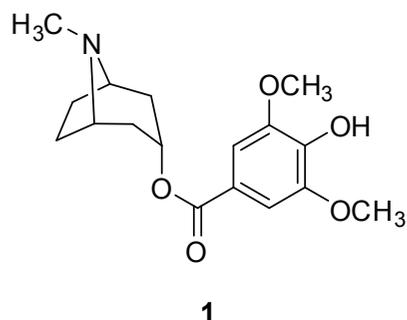
Material and Methods

Animals

Male Wistar rats (250-300 g) were used for all experiments. Animals were housed under controlled temperature (21±1 °C) and light cycle (lights on 6-18 h). Animals had free access to food (Labina®, PURINA, Brazil) and tap water. Experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee of the LTF, Federal University of Paraiba (Protocol number 0305/07).

Plant material and extraction

Erythroxylum pungens O.E. Schulz, Erythroxylaceae (EEEP), were collected near the São José de Espinharas town in Paraiba State, Brazil, in March 2007. The voucher specimen (IPA-81029) was deposited in the Herbarium Dárdano de Andrade Lima, Empresa Pernambucana de Pesquisa Agropecuária, Pernambuco State, Brazil.

Roots were air-dried at room temperature and then powdered. The powdered samples (1 kg) were de-fatted with n-hexane (2.5 L) and extracted with 75% ethanol (2.5 L) overnight in a soxhlet extractor. Part of the EtOH extract (200 g) was submitted to alkaloid extraction. The whole fraction of tertiary alkaloids (50 g) was subjected to column chromatography (CC, basic Al_2O_3).

The isolation and identification of pungencine (**1**) have been described in details elsewhere (Sena-Filho et al, 2010). EEEP or pungencine stock solution were prepared in distilled water and kept at -4 °C. EEEP or pungencine were solubilized in cremophor and diluted to the desired concentrations with distilled water just before the use. The final concentration of cremophor in the bath never exceeded 0.01% and had no effect when tested in control preparations.

Measurement of arterial pressure and heart rate in conscious rats

For blood pressure and heart rate recordings, procedures were similar to those previously described (Braga, 2010; Nunes et al., 2010). Briefly, under sodium thiopental anaesthesia (45 mg/kg, *i.p.*), rats were fitted with polyethylene catheters inserted into the lower abdominal aorta and lower vena cava through left femoral artery and vein, respectively. Both catheters were filled with heparinized saline, tunnelled subcutaneously, exteriorized and sutured at the dorsal surface of the neck. Twenty-four hours after surgical procedures, experiments were performed in conscious rats. The arterial catheter was connected to a pre-calibrated pressure transducer (Statham P23 ID; Gould, Cleveland, OH, USA). The transducer was connected to an amplifier-recorder (Model TBM-4M, WPI, Sarasota, FL, USA) and feed to a computer equipped with an analogic-to-digital converter board (CIO-DAS16/JR, Computer Boards, Inc., Mansfield, MA, USA). Using CVMS acquisition software (WPI, Sarasota, FL, USA), data were sampled every 500 Hz and stored on a CD-ROM. Beat-to-beat time waveforms were generated and processed off-line. For each cardiac cycle, the software calculated mean arterial pressure (MAP) and pulse interval (used to derive heart rate).

Cardiovascular effects of ethanolic extract from the roots of Erythroxylum pungens (EEEP)

After cardiovascular parameters had stabilized (30 min of baseline recordings), different doses of EEEP (1, 10, 30 and 60 mg/kg, *i.v.*) were randomly administered.

Vascular reactivity studies in isolated rat superior mesenteric artery rings

Rats were euthanized by stunning and bleeding. The superior mesenteric artery was removed and cleaned from connective tissue and fat. Whenever appropriated, the endothelium was removed by gently rubbing the intimal surface of the vessels. Rings (1-2 mm) were obtained and placed in physiological Tyrode's solution, maintained to 37 °C, gassed with carbogenic mixture (95% O_2 and 5% CO_2), and maintained at pH 7.4. All preparations were stabilized under a resting tension of 0.75 g for 1 h. The solution was replaced every 15 min to prevent the accumulation of metabolites (Ribeiro et al., 2010; Luciano et al., 2011). The force of contraction was isometrically recorded by a force transducer (Miobath-4, WPI, Sarasota,

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FL, USA) coupled to an amplifier-recorder (Miobath-4, WPI, Sarasota, FL, USA) and to a computer equipped with an analogic-to-digital converter board.

The presence of functional endothelium was assessed by the ability of acetylcholine (10 μM) to induce more than 90% relaxation of vessels pre-contracted with phenylephrine (10 μM). Less than 10% of relaxation to acetylcholine was taken as evidence that the vessel segments were functionally denuded of endothelium as described earlier (Ribeiro et al., 2010). In the tonic phase of the second contraction, EEEP (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 500 μg/mL) was cumulatively added to preparations until a Maximal Response for the drug administration was observed as indicated by a plateau response (approximately 10 min). The same procedure was used to observe the effects induced by pungencine (10^{-12} M to 3×10^{-4} M).

The possible effects of EEEP on KCl (80 mM) Bay k 8644-induced sustained contraction in denuded rings were also examined. To further investigate the mechanism of vasorelaxation induced by EEEP, concentration-response curves to $CaCl_2$ were constructed using endothelium-denuded rings. Briefly, the rings were pre-contracted with KCl (60 mM) to confirm tissue viability. The Tyrode's solution was replaced with depolarizing Tyrode's solution (KCl 60 mM) nominally without Ca^{2+} (for 15 min). Thereafter, concentration-response curves to $CaCl_2$ (1 μM-10 mM) were constructed in the absence or presence of EEEP. To determine whether EEEP could interfere with Ca^{2+} release from intracellular stores, the denuded rings were pre-contracted with KCl, washed and exposed to Ca^{2+}-free Tyrode's solution containing EGTA (1 mM). The rings were then stimulated with phenylephrine (10 μM). The contractions of both agonists were obtained in the absence (control) or after incubation with EEEP.

Drugs and solutions

The drugs used were: acetylcholine chloride (ACh), L-phenylephrine (Phe), S-(-)1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-[trifluoromethyl]phenyl)pyridine-3-carboxylic acid methyl ester (Bay K 8644), cremophor EL (Sigma Chemical Co., St Louis, MO, USA). Heparin sodium salt (Roche, Rio de Janeiro, RJ Brazil), sodium thiopental (Cristália, São Paulo, SP, Brazil). The other compounds were dissolved in distilled water. The composition of the Tyrode's solution used was (mM): NaCl, 158.3; KCl, 4.0; $CaCl_2$, 2.0; $MgCl_2$, 1.05; NaH_2PO_4, 0.42; $NaHCO_3$, 10.0 and glucose, 5.6.

Statistical analysis

Data are presented as mean±SEM. Statistical analyses were performed using "one-way" ANOVA followed by Tukey's post hoc test or unpaired Student's t-test when appropriate. Non-linear regressions were done by the least square method, using Graph Pad Prism TM software, version 5.0 (Graph Pad Software, Inc.).

Results

The cardiovascular responses elicited by the EEEP were evaluated in normotensive conscious rats. In five rats, baseline values for mean arterial pressure and heart rate were 109±1 mmHg and 365±5 bpm, respectively. EEEP administration (1, 10, 30 and 60 mg/kg *i.v.*, randomly) induced hypotension (-2±1, -7±0.5 -17.6±1, -24±1 ΔmmHg, n=5) followed by tachycardia (3±0.5, 7±2, 7.1±1, 10±5 Δ bpm, n=6) (Figure 1).
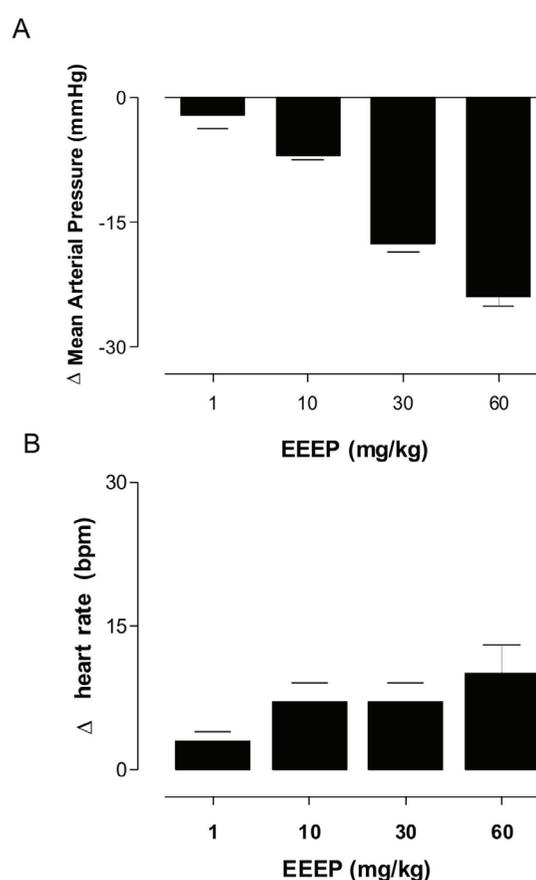
Figure 1. Changes in mean arterial pressure (MAP, A) and heart rate (HR, B) induced by the acute administration of increasing doses of EEEP (mg/kg, *i.v.*) in conscious unrestrained normotensive rats. Values are expressed as mean±SEM (n=6).

In phenylephrine-precontracted mesenteric artery rings with functional endothelium, EEEP (0.01-500 μg/mL) induced a concentration-dependent relaxation (Figure 2A) with (EC50 13.7±5.5 μg/mL, Maximal Response 92±2.6%), without any appreciable effects on maximal responses when compared to preparations lacking the endothelium (EC50 27.2±4.7 μg/mL, Maximal Response

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88.3±3.3%). The time required for reaching the maximal effect at each concentration was around 10 min.

In order to further investigate the mechanisms underlying the effects of EEEP, such effects were evaluated in the presence of a high extracellular potassium concentration solution (KCl 80 mM) (Figure 2B). Under this experimental condition, the vasorelaxant maximum effect for the EEEP was unchanged after the removal of the vascular endothelium when compared to those data obtained in mesenteric artery rings pre-contracted with phenylephrine (EC50 27.2±4.7 µg/mL, Maximal Response 88.3±3.3%, n=8 for phenylephrine 10 µM; EC50 128.2±11.2 µg/mL, Maximal Response 76.8±3.4% for KCl (80mM).

The contractions elicited by S(-)Bay K 8644 (10 µM) were very stable, making it easy to analyze the relaxing effects induced by EEEP under that experimental condition. Under those controlled conditions, EEEP produced a concentration-dependent vasorelaxation [Maximal Response= 86.4±13.6 % and an EC50 of 80.2±17.1 µg/mL (n=8)] as shown in Figure 2B.

The vasorelaxant response induced by higher concentrations of EEEP (30; 100; 300; 500 µg/mL) may be suggestive of a possible blockade of Ca^{2+} influx through the interference with both voltage- and receptor-operated channels. As shown in Figure 3, pretreatment with EEEP attenuated $CaCl_2$-induced contraction of denuded mesenteric rings exposed to Ca^{2+}-free medium containing KCl (60 mM). $CaCl_2$ induced a concentration-dependent contraction of rat mesenteric artery rings (EC50 27.2±4.7 µg/mL, Maximal Response 88.3±3.3%, n=8 for preparations without the endothelium [control]). Pre-incubation of rings with EEEP at 100-500 µg/mL, significantly reduced the Maximal Response values for $CaCl_2$ (73.2±5.1%, 53.0±11.3%, 31.7±6.3%, respectively; n = 6 for each group).

We further investigated whether EEEP could exert its vasorelaxant effects by interfering with the release of intracellular calcium via the phosphoinositide-dependent or independent pathway following receptors activation. The results presented in Figure 3 show that EEEP significantly ($p<0.05$) reduced the transient contractions induced by phenylephrine in endothelium-denuded rings in Ca^{2+}-free media containing EGTA (1 mM).

To evaluate the contribution of tropane alkaloids in the EEEP-induced vasorelaxation, we also studied the effect of pungencine (**1**) on contractions elicited by the Phe (10 µM). In isolated rat superior mesenteric rings with intact endothelium pre-contracted with phenylephrine (10 µM), increasing concentrations of pungencine (10^{-12} to 3×10^{-4} M) induced vasorelaxation in a concentration-dependent manner [(EC50 2.4 x 10^{-8} M, Maximal Response 94±4.9%, n=8)]. After removal of vascular endothelium, the relaxant response induced by pungencine

was significantly attenuated [(EC50 1.3 x 10^{-4} M, Maximal Response 65.7±4.1%] as illustrated in Figure 4.
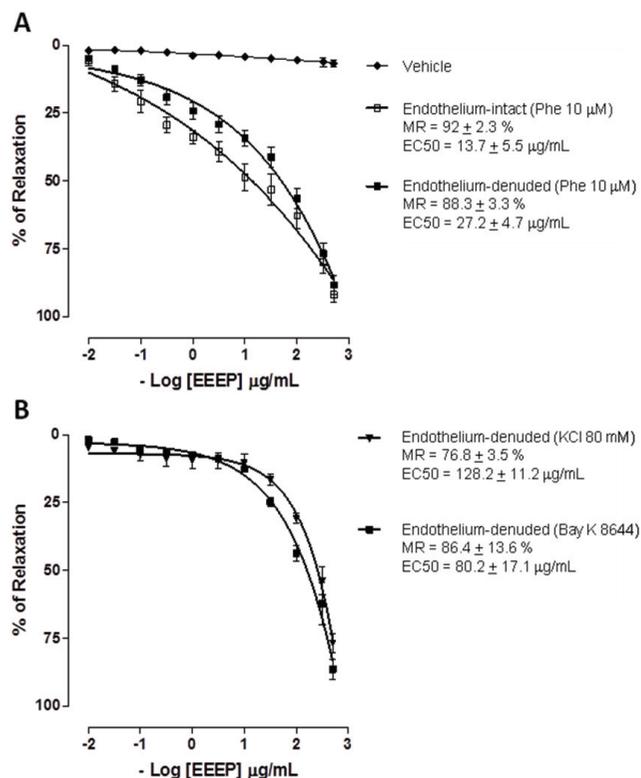

Figure 2. Responses to EEEP (µg/mL) in endothelium-intact mesenteric artery rings pre-contracted with 10 µM phenylephrine (Phe). Tracings are representative of eight similar experiments. A. Concentration-response curves showing the relaxant effect of EEEP (0.01 to 500 µg/mL) and vehicle (♦). The response is expressed as a percentage relaxation of the phenylephrine-induced contraction (100% represented relax completely). Each data point and vertical bar represents the mean and the SEM from eight experiments. *p< 0.05 indicate significant differences between EC50 values of the endothelium-intact (□) and the endothelium-denuded (■). B. Endothelium-denuded – Phe 10µM (▼) or Endothelium-denuded - KCl 80 mM or Endothelium-denuded – Bay K 8644 (■). MR=Maximal Response.

Discussion

The major findings of this work were that the EEEP induced dose-dependent hypotension and tachycardia in conscious rats and vasorelaxation in mesenteric artery ring preparations in vitro. The changes in blood pressure seem to be due to a decrease in the vascular resistance as discussed in details below. On the other hand, the tachycardia seems to be mediated by the baroreflex in response to the fall in blood pressure.
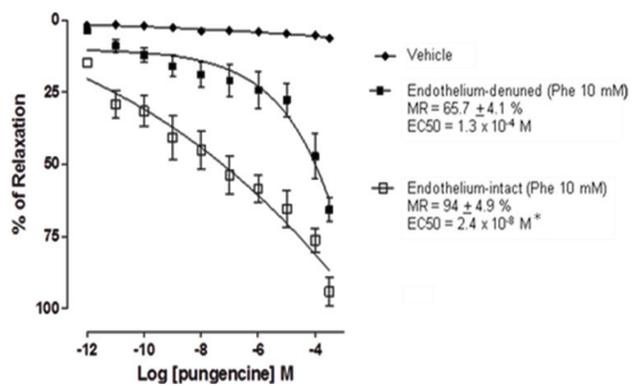
In order to investigate the direct effect of EEEP on the vasculature, independent of neurohumoral influences, we performed experiments in rat isolated
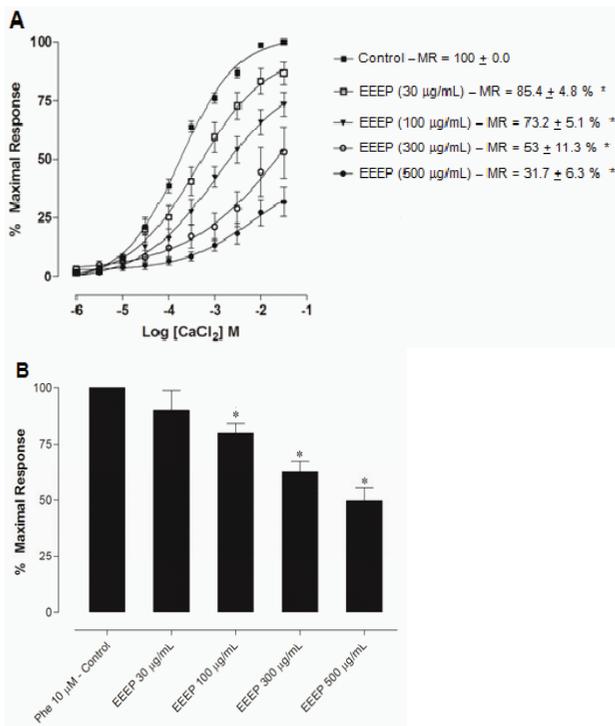
Figure 3. Effects of EEEP on CaCl$_2$-induced contraction in endothelium-denuded mesenteric artery rings. Concentration-response curves for CaCl$_2$ were determined in Ca^{2+}-free solution containing KCl (60 mM). A. The curves were determined in the absence (control) (■) and after incubation with EEEP 30 µg/mL (□); 100 µg/mL (▼); 300 µg/mL (▣); 500 µg/mL (●). B. Effect of EEEP (30, 100, 300, 500 µg/mL) on phenylephrine (10 µM) induced transient contractions in endothelium-denuded mesenteric artery rings in Ca^{2+}-free medium containing EGTA (1 mM). *Significantly different from control (*p*<0.05). MR=Maximal Response.

Figure 4. Responses to pungencine (M) in endothelium-intact mesenteric artery rings pre-contracted with 10 µM phenylephrine (Phe). Tracings are representative of eight similar experiments. Concentration-response curves showing the relaxant effect of pungencine (10^{-12} to 10^{-4} M) and vehicle (♦). The response is expressed as a percentage relaxation of the phenylephrine-induced contraction (100% represented relax completely). Each data point and vertical bar represents the mean and the SEM from eight experiments. *p*<0.05 indicate significant differences between EC50 values of the endothelium-intact (□) and the endothelium-denuded (■). MR=Maximal Response.

superior mesenteric artery rings using different contracting agents. In intact rings pre-contracted with Phe (10 µM), an alpha-1 receptor agonist, EEEP induced a remarkable relaxant effect in a concentration-dependent manner. After endothelium removal, this effect was unchanged (Figure 2A). The mechanism by which these effects occur seems to be independent of endothelium-derivative relaxant factors (EDRF).

We also evaluated the effects induced by EEEP in an experimental condition where extracellular K$^+$ was raised from 4 to 80 mM, showing that EEEP induced a concentration-dependent and endothelium-independent relaxant activity on Phe- or KCl-pre-contracted mesenteric artery rings, suggesting that vasodilatations in both case were produced by interfering with a common pathway, which seems to involve calcium. Contractions of vascular smooth muscle cells induced by KCl (80 mM) rely almost exclusively on Ca^{2+} influx through activation of voltage-sensitive channels, (Hirata et al., 1998), whereas contractions induced by Phe are mediated by an increase in Ca^{2+} influx through both receptor-operated channels

(Lee et al., 2001) and voltage-sensitive channels (Eckert et al., 2000). An increase in free cytoplasmic Ca^{2+} levels is required for excitation-contraction coupling of vascular smooth muscle (Gollasch et al., 1998). The increase in cytosolic calcium is due to activation of Ca^{2+} permeable ion channels on the plasma membrane and release of calcium from the sarcoplasmic reticulum (Catterall, 2010).

One possibility for increasing the intracellular calcium concentration is via activation of L-type calcium channels in the membrane. Our results demonstrate that the vasorelaxation induced by EEEP in mesenteric artery rings lacking the endothelium was unaffected by the activation of L-type calcium channels using Bay K 8644.

We further investigated the possible link between EEEP-induced vasorelaxation and the inhibition of Ca^{2+} influx from the extracellular medium. We observed that the CaCl$_2$-induced contractions in depolarizing medium nominally without Ca^{+2} were inhibited by EEEP (30-500 µg/mL) in a concentration dependent manner (Figure 3A). In addition, in Ca^{+2}-free medium, EEEP (30, 100, 300, 500µg/ml) significantly inhibited the transient contraction induced by phenylephrine (10 µM) (Figure 3).

Taken together, these results support the notion that EEEP can block Ca^{2+} influx through Ca^{2+} channels present in the vascular smooth muscle cells and suggest that the endothelium-independent vasorelaxant effect induced by EEEP is probably due to the blockade of the Ca^{+2} influx through voltage-dependent calcium channels as well as inhibition of calcium release from intracellular stores sensitive to IP3. This interpretation is in agreement

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with findings reported by others (Medeiros et al., 2009; Bastos et al., 2010; Cavalcante et al., 2011). However, this result does not rule out the possibility that EEEP interferes with the sarco-endoplasmatic reticulum Ca^{2+}-ATPases. This interesting possibility is still matter for further investigation.

EEEP contains many constituents, being the tropane alkaloid (pungencine (**1**)) its major component (Sena-Filho et al., 2010). For comparison, after isolation and identification, we tested the effects elicited by the administration of pungencine on mesenteric artery rings. Our results demonstrated that pungencine (10^{-12} a 3×10^{-4} M) induced a marked relaxant effect in a concentration manner in intact rings pre-contracted with Phe (10 µM), which was attenuated after the removal of the functional endothelium (Figure 4). Therefore, the vasorelaxant response induced by the EEEP is due, at least in part, to pungencine. However, there is no information in the literature about the effects of this compound on vascular smooth muscle cells. In light of this new evidence, further studies aiming to identify the mechanisms underlying the vasorelaxant effects of this compound are needed.

In conclusion, using combined *in vivo* and *in vitro* approaches we demonstrated that EEEP lowers arterial blood pressure in normotensive rats, probably secondary to a decrease in the peripheral vascular resistances. Furthermore, the vasorelaxant action of EEEP is partly mediated by the blockade of extracellular Ca^{2+} influx by interfering with both voltage-dependent and receptor-operated channels and by inhibition of Ca^{2+} release from inositol-1,4,5-triphosphate (IP3) sensitive stores in vascular smooth muscle cells. In addition, pungencine was found to elicit vasorelaxation, requiring further studies to unravel its potential mechanisms on the cardiovascular system.

Acknowledgements

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