



Cardiovascular Pharmacology

The semi-synthetic kaurane *ent*-16 α -methoxykauran-19-oic acid induces vascular relaxation and hypotension in rats

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ARTICLE INFO

Article history:

Received 9 September 2010

Received in revised form 16 March 2011

Accepted 6 April 2011

Available online 27 April 2011

Keywords:

ent-16 α -methoxykauran-19-oic acid

Relaxation

Rat aorta

Diterpene

Ca²⁺

ABSTRACT

The present work investigates the mechanisms involved in the vasorelaxant effect of *ent*-16 α -methoxykauran-19-oic acid (KA-OCH₃), a semi-synthetic derivative obtained from the kaurane-type diterpene *ent*-kaur-16-en-19-oic acid (kaurenoic acid). Vascular reactivity experiments were performed in aortic rings isolated from male Wistar rats using standard muscle bath procedures. The cytosolic calcium concentration ([Ca²⁺]_c) was measured by confocal microscopy using the fluorescent probe Fluo-3 AM. Blood pressure measurements were performed in conscious rats. KA-OCH₃ (10, 50 and 100 μ mol/l) inhibited phenylephrine-induced contraction in either endothelium-intact or endothelium-denuded rat aortic rings. KA-OCH₃ also reduced CaCl₂-induced contraction in a Ca²⁺-free solution containing KCl (30 mmol/l) or phenylephrine (0.1 μ mol/l). KA-OCH₃ (0.1–300 μ mol/l) concentration-dependently relaxed endothelium-intact and endothelium-denuded aortas pre-contracted with either phenylephrine or KCl, to a greater extent than kaurenoic acid. Moreover, a Ca²⁺ mobilisation study showed that KA-OCH₃ (100 μ mol/l) inhibited the increase in Ca²⁺ concentration in smooth muscle and endothelial cells induced by phenylephrine or KCl. Pre-incubation of intact or denuded aortic rings with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μ mol/l), 7-nitroindazole (100 μ mol/l), wortmannin (0.5 μ mol/l) and 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μ mol/l) produced a rightward displacement of the KA-OCH₃ concentration–response curve. Intravenous administration of KA-OCH₃ (1–10 mg/kg) reduced mean arterial blood pressure in normotensive rats. Collectively, our results show that KA-OCH₃ induces vascular relaxation and hypotension. The mechanisms underlying the cardiovascular actions of KA-OCH₃ involve blockade of Ca²⁺ influx and activation of the NO-cGMP pathway.

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1. Introduction

Diterpenoids form a large class of plant-derived secondary metabolites that possess a wide spectrum of important biological activities. Many reports have shown that several classes of diterpenoids exert significant cardiovascular effects (Hipólito et al., 2009;

Tirapelli et al., 2004a,b; Tirapelli et al., 2008a; Tirapelli et al., 2010). The rationale for the study of the cardiovascular actions of diterpenes is based on the fact that many medicinal plants contain diterpenoids and that their cardiovascular activity can most likely be attributed to these compounds (Baccelli et al., 2005; El Bardai et al., 2003).

Somova et al. (2001) showed that the kaurane *ent*-kaur-16-en-15-one-19-oic acid reduces vascular resistance via inhibition of extracellular Ca²⁺ influx. Similarly, *ent*-kaur-16-en-19-oic acid (kaurenoic acid) inhibited extracellular Ca²⁺ influx and induced vascular relaxation (Tirapelli et al., 2002; Tirapelli et al., 2004a). The relaxant effect of kaurenoic acid also involves the activation of the nitric oxide (NO)-cGMP pathway (Tirapelli et al., 2004a). Intravenous administration of *ent*-kaur-16-en-19-oic acid and *ent*-kaur-16-en-15-one-19-oic acid at 10 mg/kg in normotensive rats produced an immediate decrease in systolic blood pressure (by 17 and 18%, respectively), without changing diastolic blood pressure, and a significant decrease

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in heart rate (by 20 and 55%, respectively) (Somova et al., 2001). Similar results were obtained in our laboratory with *ent*-kaur-16-en-19-oic acid (15 mg/kg, i.v.), which was found to decrease mean arterial pressure in conscious normotensive rats (Tirapelli et al., 2008b).

It is interesting to note that the mechanisms underlying the cardiovascular action of kaurane-type diterpenes involve multiple actions on various targets (Tirapelli et al., 2004a). Structural differences amongst diterpenes could explain these diverse mechanisms of action; we previously demonstrated that slight modifications in the chemical structure of these compounds alter their vascular effects (Ambrosio et al., 2004; Müller et al., 2003; Tirapelli et al., 2005). In fact, Zamilpa et al. (2002) demonstrated that the inhibitory effect displayed by *ent*-beyer-15-en-19-oic acid on electrically induced contractions of guinea-pig ileum is more pronounced than that of kaurenoic acid. The structure of this compound differs slightly from that of kaurenoic acid because of changes in ring D, such as the relative stereochemistry, the presence of a double bond between C-15 and C-16, the attachment of a methyl group to C-13 and the lack of an exocyclic methylene group at C-16. These results provided new insight into the structure-activity relationship of the kauranes because it was shown that these changes in ring D account for improvements in the antispasmodic effect elicited by kaurane-type diterpenes. Based on such findings, we hypothesised that structural alterations could improve the cardiovascular activity of kaurane-type diterpenes. To test our hypothesis, kaurenoic acid, a naturally occurring diterpene, was chemically modified. The semi-synthetic derivative obtained, the diterpene *ent*-16 α -methoxykauran-19-oic acid (KA-OCH₃), differs from kaurenoic acid because of the insertion of an α -methoxy group in ring D at C-16. This compound was tested for its cardiovascular activity.

2. Materials and methods

2.1. Synthesis of KA-OCH₃

Eight drops of concentrated H₂SO₄ were added to a solution of *ent*-kaur-16(17)-en-19-oic acid (kaurenoic acid; 200.0 mg; 0.66 mmol) in methanol (10 ml). Kaurenoic acid was isolated from the dried leaves of *Mikania hirsutissima* (1 kg), which were purchased from Nutri Comércio de Ervas LTDA, São Paulo, SP, Brazil. The mixture was stirred at 25 °C for 18 h and then poured into water (40 ml) and extracted with ethyl acetate (3 × 20 ml). The combined organic layer was dried over MgSO₄, filtered and concentrated in a rotary evaporator to yield 150.0 mg of *ent*-16 α -methoxykauran-19-oic acid (KA-OCH₃). This compound was purified using “flash” chromatography (Still et al., 1978), with *n*-hexane/ethyl acetate (9:1 v/v) as the mobile phase, and identified by means of spectrometric analysis and comparison with data from the literature (Boeck et al., 2005). Kaurenoic acid and KA-OCH₃ were evaluated by TLC using different solvent systems. According to these results and to their ¹H and ¹³C NMR spectra, we concluded that the purity of these compounds was suitable for biological assays. Analysis of the chemical structure of both diterpenes showed that KA-OCH₃ differs from kaurenoic acid only by the insertion of an α -methoxy group at C-16 (Fig. 1).

2.2. Vessel ring preparation

Male Wistar rats weighting between 200 and 250 g (50–60 days old) were anaesthetised and killed by aortic exsanguination in accordance with the Ethical Animal Committee of the Campus of Ribeirão Preto – University of São Paulo. The thoracic aorta was quickly removed, cleaned of adherent connective tissues and cut into rings (5–6 mm in length). Two stainless-steel stirrups were passed through the lumen of each ring. One stirrup was connected to an isometric force transducer (TRI201; Panlab, Spain) to measure tension

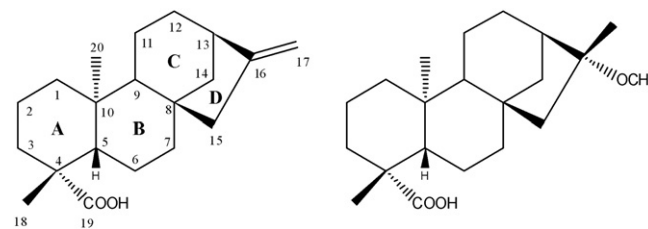


Fig. 1. Chemical structure of *ent*-kaur-16(17)-en-19-oic acid (kaurenoic acid; left) and *ent*-16 α -methoxykauran-19-oic acid (KA-OCH₃; right).

in the vessels. The rings were placed in a 5 ml organ chamber that contained Krebs solution, gassed with 95% O₂/5% CO₂ maintained at 37 °C. The composition of Krebs solution was as follows (mmol/l): NaCl, 118.0; KCl, 4.7; KH₂PO₄, 1.2; MgSO₄, 1.2; NaHCO₃, 15.0; Glucose, 5.5; CaCl₂, 2.5. The rings were stretched until they reached a basal tension of 1.5 g, which was determined by length-tension relationship experiments and were then allowed to equilibrate for 60 min; during this time, the bath fluid was changed every 15–20 min. For some rings, the endothelium was removed mechanically by gently rolling the lumen vessel on a thin wire. Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (1 μ mol/l) in the presence of contractile tone induced by phenylephrine (0.1 μ mol/l). For studies of endothelium-intact vessels, a ring was discarded if relaxation with acetylcholine was not 80% or greater. For studies of endothelium-denuded vessels, a ring was discarded if there was any degree of relaxation.

2.3. Determination of the time-sensitive effects of KA-OCH₃

Endothelium-intact rings were contracted with KCl at 30 mmol/l (control) and then washed out and pre-incubated with KA-OCH₃ (10, 50 or 100 μ mol/l) for 30 or 60 min. Subsequently, a new stimulation was performed with KCl at 30 mmol/l on the same ring; each ring served as its own control. Vessel rings from the same animal that were not exposed to the diterpene served as time controls.

2.4. Effect of KA-OCH₃ on contractions induced by phenylephrine and CaCl₂

After equilibration, cumulative concentration–response curves for phenylephrine (10 nmol/l to 10 μ mol/l) were determined. The curves were obtained in endothelium-intact and endothelium-denuded rings by a stepwise increase in the concentration of phenylephrine. Additions were made as soon as a steady response was obtained at the preceding concentration. The curves for phenylephrine were determined without pre-incubation (control group) or after a 30-min incubation with KA-OCH₃ (10, 50 or 100 μ mol/l).

To assess the effects of KA-OCH₃ on CaCl₂-induced contractions, endothelium-denuded rings were first contracted with phenylephrine (0.1 μ mol/l) in a Ca²⁺-free solution containing EGTA (1 mmol/l) for approximately 90 min to deplete the intracellular Ca²⁺ stores and then rinsed in a Ca²⁺-free solution (without EGTA) containing KCl (30 mmol/l) or phenylephrine (0.1 μ mol/l) (Hipolito et al., 2009). The cumulative concentration–response curves for CaCl₂ (0.05–2 mmol/l) were obtained without pre-incubation (control group) or after a 30 min incubation period with KA-OCH₃ (10, 50 or 100 μ mol/l).

2.5. Influence of KA-OCH₃ on Ca²⁺ release from intracellular stores sensitive to phenylephrine and caffeine

To investigate whether KA-OCH₃ could interfere with Ca²⁺ release from intracellular stores, the normal Krebs solution was replaced with a Ca²⁺-free solution containing EGTA (1 mmol/l). The rings were exposed to this solution for 1 min (David et al., 2002) and were then

stimulated with phenylephrine (1 $\mu\text{mol/l}$) or caffeine (30 mmol/l). The contractions induced by both agonists were obtained without pre-incubation (control group) or after a 30-min incubation period with KA-OCH₃ (10, 50 or 100 $\mu\text{mol/l}$).

2.6. Effect of KA-OCH₃ on aortic rings contracted with phenylephrine or KCl

In another set of experiments, steady tension was evoked by phenylephrine (concentrations of 0.1 $\mu\text{mol/l}$ for endothelium-intact rings and 0.03 $\mu\text{mol/l}$ for endothelium-denuded rings were used to induce contractions of similar magnitude), and KA-OCH₃ was then added in a stepwise fashion (0.1–300 $\mu\text{mol/l}$). The effect of KA-OCH₃ on KCl-induced sustained contraction (30 mmol/l) in intact or denuded rings was also examined. For comparison, the effects of kaurenoic acid (1–450 $\mu\text{mol/l}$) and verapamil (1 nmol/l to 100 $\mu\text{mol/l}$), a blocker of voltage-dependent Ca²⁺ channels on the contractions induced by phenylephrine and KCl in endothelium-intact and endothelium-denuded rings were also evaluated. The cumulative concentration–response curves were obtained using stepwise increases in the concentrations of KA-OCH₃, kaurenoic acid or verapamil. Additions were made as soon as a steady response was obtained at the preceding concentration.

To investigate the possible mechanism(s) responsible for KA-OCH₃-induced relaxation, endothelium-intact or denuded rings were contracted with phenylephrine (0.1 $\mu\text{mol/l}$ and 0.03 $\mu\text{mol/l}$, respectively) 30 min after being incubated with one of the following drugs: N^G-Nitro-L-arginine methyl ester (L-NAME, 100 and 300 $\mu\text{mol/l}$), wortmannin (0.5 and 1 $\mu\text{mol/l}$), 7-nitroindazole (100 $\mu\text{mol/l}$), N-([3-(Aminomethyl)phenyl]methyl)ethanimidamide dihydrochloride (1400 W, 1 $\mu\text{mol/l}$), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 and 3 $\mu\text{mol/l}$), indomethacin (10 $\mu\text{mol/l}$) and tetraethylammonium (1 mmol/l). Relaxation was expressed as percentage change from the phenylephrine-contracted levels. Because L-NAME and ODQ enhanced phenylephrine-induced contraction, the rings with intact endothelium exposed to these compounds were pre-contracted with phenylephrine (0.03 $\mu\text{mol/l}$) to induce a magnitude of contraction similar to that found in the intact rings not exposed to the inhibitors.

2.7. Confocal microscopy and image analysis

To image arterial cross sections, the rings (150 μm thick) were placed vertically onto a coverslip covered with poly-L-lysine. The tissue was loaded with the fluorescent Ca²⁺ dye Fluo-3 AM by incubation in normal Krebs solution containing 10 $\mu\text{mol/l}$ Fluo-3 AM (Hipólito et al., 2009) for 1 h at room temperature. After washing, the segment was placed in a chamber with a volume of 1 ml. The chamber was placed on the stage of a confocal microscope, and images were taken from the bottom of the chamber through a water-immersion objective (63 \times). Ca²⁺ images of the aortic ring segment were taken sequentially once every 1.5 s in Krebs solution (pH 7.4). The cytosolic Ca²⁺-concentration ([Ca²⁺]_c) was assessed by confocal scanning laser microscope (Leica TCS SP5). Fluo-3 AM fluorescence was excited with the 488-nm line of an argon ion laser, and the emitted fluorescence was measured at 510 nm. Time course software was used to capture images of the cells at intervals of 1.5 s (xyt) in Live Data Mode acquisition at 512 \times 512 pixel and 700 Hz. The protocol was designed to measure [Ca²⁺]_c in slices of rat aorta with intact endothelium. Ca²⁺ concentration was measured in vascular smooth muscle and endothelial cells in the absence and in the presence of phenylephrine (10 $\mu\text{mol/l}$) or KCl (60 mmol/l). In another set of experiments, [Ca²⁺]_c was measured in slices of rat aorta that were pre-incubated with verapamil (1 $\mu\text{mol/l}$, a voltage-dependent Ca²⁺ channels blocker) or KA-OCH₃ (100 $\mu\text{mol/l}$). Verapamil and KA-OCH₃ were incubated for 30 min before the application of phenylephrine or KCl. Using the LSCM software, the intensities of the maximum and minimum

intracellular fluorescence were measured. In this way, the percentage of the difference in fluorescence intensity (% ΔFI), which reflects the increase in [Ca²⁺]_c, was obtained for each sample in relation to the control (100%) using the following equation:

$$\% \Delta F = (F - F_0 / F_0) \times 100 \quad (1)$$

Where F₀ = the basal fluorescence intensity of Fluo-3 AM and F = the fluorescence intensity of Fluo-3 AM after stimulation of the aortic ring with phenylephrine (10 $\mu\text{mol/l}$) or KCl (60 mmol/l) in the absence or presence of verapamil (1 $\mu\text{mol/l}$) or KA-OCH₃ (100 $\mu\text{mol/l}$). The fluorescence signal used to calculate [Ca²⁺]_c was measured over the whole cell and was averaged.

2.8. Blood pressure experiments

One day before the experiments, the rats were anaesthetised with tribromoethanol, and a catheter (a 4 cm segment of PE-10 heat-bound to a 13 cm segment of PE-50, Clay Adams, Parsippany, NJ, USA) was inserted into the abdominal aorta through the femoral artery for blood pressure and heart rate recording. A second catheter was implanted into the femoral vein for intravenous administration of drugs. Both catheters were implanted under the skin and exited at the animal's back. During the experiment, freely moving rats were kept in individual cages, and mean arterial pressure and heart rate were recorded using an HP-7754A amplifier (Hewlett Packard, USA) connected to a signal acquisition board (MP-100, BIOPAC, USA) and processed by a computer. Dose–response curves for KA-OCH₃ and kaurenoic acid were obtained by bolus intravenous injection of the diterpenes (1–10 mg/kg). Blood pressure responses were calculated based on the average mean blood pressure calculated at the response's plateau.

2.9. Drugs

The following drugs were used: phenylephrine hydrochloride, acetylcholine hydrochloride, verapamil, ODQ, 7-nitroindazole, 1400 W, indomethacin, caffeine, wortmannin, L-NAME, tetraethylammonium (Sigma-Aldrich, St. Louis, MO, USA), potassium chloride and calcium chloride (Synth, São Paulo, Brazil). KA-OCH₃, kaurenoic acid, ODQ, wortmannin and 7-nitroindazole were prepared as stock solutions in dimethyl sulfoxide (DMSO). Indomethacin was dissolved in Tris buffer (pH 8.4). The other drugs were dissolved in distilled water. The bath concentration of DMSO did not exceed 0.5%, which was shown to have no effect per se on the basal tonus of the preparations or on the agonist-mediated contraction or relaxation. For the in vivo experiments, KA-OCH₃ was diluted in 10% DMSO and then in saline. The concentration of DMSO in the final solution had no effects per se on basal cardiovascular parameters (mean arterial pressure and heart rate), as previously observed (Tirapelli et al., 2008c).

2.10. Statistical analysis

To study the effect of KA-OCH₃ on contraction/relaxation, two pharmacological parameters were analysed: E_{max} (maximal effect generated by the agonist) and pD₂ (–log EC₅₀). The following equation was used to calculate E_{max} and pD₂:

$$Y = \text{Bottom} + [\text{Top} - \text{Bottom}] / [1 + 10^{\log EC_{50} - X}]$$

where X is the logarithm of the concentration and Y is the response. A plot of Y versus X going from the bottom to the top has a sigmoid shape. Blood pressure results were expressed as Δ mean arterial pressure (mm Hg) and Δ heart rate (bpm). Results were expressed as means \pm standard errors of the mean (S.E.M.). Statistical analysis of

the vascular reactivity experiments (E_{\max} and pD_2 values) was performed using one-way analysis of variance (ANOVA). The same analysis was applied to the blood pressure experiments. Post-hoc comparisons were performed after ANOVA analysis using a Newman–Keuls multiple comparison test as indicated in the text and tables. For all analyses, P values of less than 0.05 were considered significant.

3. Results

3.1. Determination of the time-dependent effects of KA-OCH₃

No differences in the contraction induced by KCl (30 mmol/l) at different time points were detected (data not shown). KA-OCH₃ inhibited the contraction induced by KCl in a concentration-dependent manner. However, no differences were found between the two periods of incubation (Table 1), indicating that KA-OCH₃ achieved its maximal inhibitory action at 30 min (equilibrium period). Based on these findings, the 30-min period of incubation was chosen to study the mechanisms underlying the vascular action displayed by KA-OCH₃.

3.2. Effect of KA-OCH₃ on contractions induced by phenylephrine and CaCl₂

The chemical structures of KA-OCH₃ and kaurenoic acid are represented in Fig. 1. The E_{\max} values for phenylephrine in both endothelium-intact and endothelium-denuded rings were reduced in the presence of KA-OCH₃ at 10, 50 and 100 $\mu\text{mol/l}$. KA-OCH₃ reduced the pD_2 values for phenylephrine in both endothelium-intact and endothelium-denuded rings (Table 2).

As shown in Fig. 2, pre-treatment of the rings with KA-OCH₃ attenuated CaCl₂-induced contraction in denuded rat aortas exposed to Ca²⁺-free medium containing KCl. Pre-incubation of the rings with KA-OCH₃ at 10, 50 or 100 $\mu\text{mol/l}$ significantly reduced the E_{\max} values for CaCl₂ (Table 3). CaCl₂-induced contraction in Ca²⁺-free medium containing phenylephrine was attenuated by KA-OCH₃ at doses of 50 and 100 $\mu\text{mol/l}$ (Fig. 2, Table 3).

3.3. Influence of KA-OCH₃ on Ca²⁺ release from intracellular stores

The results presented in Table 4 show that the contractions induced by phenylephrine or caffeine in Ca²⁺-free solution were not affected by KA-OCH₃.

3.4. Effect of KA-OCH₃ on aortic rings pre-contracted with phenylephrine or KCl

KA-OCH₃, at concentrations ranging from 0.1 to 300 $\mu\text{mol/l}$, significantly reduced the sustained contractions induced by phenylephrine and KCl in a concentration-dependent manner (Fig. 3). The E_{\max} values (percentage of relaxation) for the relaxant effect of KA-OCH₃ in

Table 1

Percentage inhibition induced by KA-OCH₃ on KCl-induced contraction of endothelium-intact rat aorta after different periods of incubation.

Minutes	KA-OCH ₃		
	10 $\mu\text{mol/l}$	50 $\mu\text{mol/l}$	100 $\mu\text{mol/l}$
30	20.1 \pm 1.1 ^a (5)	47.5 \pm 3.4 ^{a,b} (4)	68.7 \pm 2.8 ^{a,b,c} (4)
60	17.9 \pm 1.2 ^a (4)	55.2 \pm 2.6 ^{a,b} (4)	70.3 \pm 7.1 ^{a,b,c} (4)

Number between parentheses indicates the number of animals. Values are means \pm S.E.M. The rings were initially stimulated with KCl 30 mmol/l (control group, 100% contraction) and a second stimulation was performed after incubation with KA-OCH₃.

^a Significant difference from control (0% inhibition).

^b Significant difference from KA-OCH₃ at 10 $\mu\text{mol/l}$.

^c Significant difference from KA-OCH₃ at 50 $\mu\text{mol/l}$ (ANOVA followed by Newman–Keuls multiple comparison test, $p < 0.05$).

Table 2

Effect of KA-OCH₃ on the E_{\max} (g) and pD_2 values for phenylephrine in endothelium-intact (Endo+) or denuded (Endo-) aortic rings.

KA-OCH ₃ [$\mu\text{mol/l}$]	E_{\max}		pD_2	
	Endo+	Endo-	Endo+	Endo-
0	1.45 \pm 0.10 (10)	1.76 \pm 0.10 (6)	7.84 \pm 0.28	8.30 \pm 0.16
10	1.25 \pm 0.10 ^a (6)	1.31 \pm 0.15 ^a (8)	7.48 \pm 0.23	7.74 \pm 0.30
50	1.00 \pm 0.06 ^a (11)	1.10 \pm 0.09 ^a (5)	7.00 \pm 0.09 ^a	7.53 \pm 0.17 ^a
100	0.82 \pm 0.07 ^{a,b} (11)	1.00 \pm 0.18 ^{a,b} (5)	6.95 \pm 0.07 ^{a,b}	7.47 \pm 0.10 ^a

Number between parentheses indicates the number of animals. Values are means \pm S.E.M.

^a Compared to control group.

^b Compared to KA-OCH₃ 10 $\mu\text{mol/l}$ (ANOVA followed by Newman–Keuls multiple comparison test, $P < 0.05$).

endothelium-intact and endothelium-denuded rings pre-contracted with phenylephrine were not significantly different (80.6 \pm 2.8%, $n = 10$ and 80.8 \pm 6.4%, $n = 6$, respectively). Similarly, no differences were found in the pD_2 values for KA-OCH₃ in intact or denuded rings (5.29 \pm 0.10 and 5.08 \pm 0.07; respectively). In the arteries pre-contracted with KCl, there was no difference between the E_{\max} values for KA-OCH₃ in intact or denuded rings (85.4 \pm 3.9%; $n = 6$ and 85.1 \pm 8.0%, $n = 6$, respectively). The E_{\max} values for KA-OCH₃ in the rings pre-contracted with KCl were not different from those found in phenylephrine-pre-contracted rings. The pD_2 values for KA-OCH₃ in intact and denuded rings pre-contracted with KCl were not significantly different (5.27 \pm 0.04 and 5.22 \pm 0.11, respectively).

The relaxation induced by kaurenoic acid (1–450 $\mu\text{mol/l}$) in endothelium-intact and endothelium-denuded rings pre-contracted with phenylephrine was not significantly different (72.3 \pm 3.3%, $n = 7$ and 80.1 \pm 3%, $n = 5$, respectively) from that found for KA-OCH₃ (80.6 \pm 2.8%, $n = 10$ and 80.8 \pm 6.4%, $n = 6$, respectively). However, differences were found in the pD_2 values for kaurenoic acid in intact or denuded rings (4.4 \pm 0.14 and 4.5 \pm 0.12; respectively) when compared to KA-OCH₃ (5.29 \pm 0.10 and 5.08 \pm 0.07; respectively) ($P < 0.05$, ANOVA followed by Newman–Keuls multiple comparison test). In the arteries

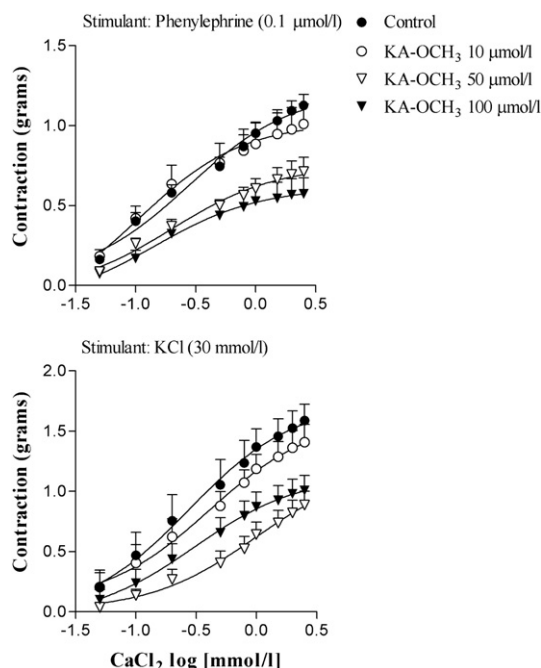


Fig. 2. Effect of KA-OCH₃ on CaCl₂-induced contractile response in endothelium-denuded aortic rings. Concentration–response curves for CaCl₂ were determined in Ca²⁺-free solution containing phenylephrine (0.1 $\mu\text{mol/l}$) or KCl (30 mmol/l). The curves were determined in the absence (control) or after a 30 min period of incubation with KA-OCH₃ (10, 50 or 100 $\mu\text{mol/l}$).

Table 3
Effect of KA-OCH₃ on CaCl₂-induced contraction in Ca²⁺-free solution containing phenylephrine (0.1 μmol/l) or KCl (30 mmol/l).

KA-OCH ₃ [μmol/l]	Stimulant: phenylephrine		Stimulant: KCl	
	E _{max} (grammes)	pD ₂	E _{max} (grammes)	pD ₂
0	1.14 ± 0.10 (5)	0.64 ± 0.08	1.58 ± 0.13 (6)	0.66 ± 0.14
10	1.00 ± 0.12 (7)	0.79 ± 0.11	1.40 ± 0.14 (6)	0.71 ± 0.15
50	0.72 ± 0.10 ^a (7)	0.79 ± 0.10	1.00 ± 0.12 ^{a,b} (5)	0.64 ± 0.15
100	0.58 ± 0.10 ^a (6)	0.74 ± 0.13	0.88 ± 0.11 ^{a,b} (6)	0.37 ± 0.11 ^{a,b,c}

Number between parentheses indicates the number of animals. Values are means ± S.E.M.

^a Compared to control group.

^b Compared to KA-OCH₃ 10 μmol/l.

^c Compared to KA-OCH₃ 50 μmol/l (ANOVA followed by Newman-Keuls multiple comparison test, P < 0.05).

pre-contracted with KCl no difference was found between the E_{max} values for kaurenoic acid (74.5 ± 4.8%; n = 8 and 78.2 ± 1.3%, n = 8, respectively) and KA-OCH₃ in intact and denuded rings (85.4 ± 3.9%; n = 6 and 85.1 ± 8.0%, n = 6, respectively). The pD₂ values for kaurenoic acid in intact and denuded rings pre-contracted with KCl (4.3 ± 0.11 and 4.2 ± 0.1, respectively) were significantly different from those found for KA-OCH₃ (5.27 ± 0.04 and 5.22 ± 0.11, respectively) (P < 0.05, ANOVA followed by Newman-Keuls multiple comparison test).

Fig. 3 shows that the relaxation induced by verapamil in endothelium-intact (98.8 ± 2.4%, n = 6) and endothelium-denuded (100.4 ± 2.3%, n = 8) rings pre-contracted with phenylephrine was significantly different from that observed with KA-OCH₃ (80.6 ± 2.8%, n = 10 and 80.8 ± 6.4%, n = 6, respectively) (P < 0.05, ANOVA followed by Newman-Keuls multiple comparison test). The pD₂ values for verapamil in endothelium-intact (pD₂ = 6.3 ± 0.1) and endothelium-denuded (pD₂ = 6.2 ± 0.2) rings were higher than those obtained using KA-OCH₃ (5.29 ± 0.10 and 5.08 ± 0.07; respectively) (P < 0.05, ANOVA followed by Newman-Keuls multiple comparison test). Verapamil also exerted a more pronounced relaxation in intact (102.1 ± 1.1%, n = 7) and denuded (107.1 ± 2.3%, n = 7) KCl-pre-contracted aortic rings when compared with KA-OCH₃ in rings with or without endothelium (85.4 ± 3.9%; n = 6 and 85.1 ± 8.0%, n = 6, respectively). Again, the pD₂ values found for verapamil in intact and denuded rings (pD₂ = 6.7 ± 0.1; 6.9 ± 0.2) were higher than those obtained for KA-OCH₃ in endothelium-intact or endothelium-denuded rings (5.27 ± 0.04 and 5.22 ± 0.11, respectively) (P < 0.05, ANOVA followed by Newman-Keuls multiple comparison test).

3.5. Effect of several inhibitors on KA-OCH₃-induced relaxation in phenylephrine pre-contracted rings

There was a rightward displacement of the concentration-response curve for KA-OCH₃ in endothelium-intact and endothelium-denuded rings in the presence of L-NAME (a non-selective NOS inhibitor), 7-nitroindazole (a selective nNOS inhibitor), wortmannin (a selective PI3K inhibitor) and ODQ (a selective guanylyl cyclase inhibitor) (Table 5). At high concentrations, L-NAME and ODQ reduced the maximal relaxation induced by KA-OCH₃. On the other hand, 1400 W

Table 4
Effect of KA-OCH₃ on phenylephrine (1 μmol/l) and caffeine (30 mmol/l)-induced contraction (g) in Ca²⁺-free medium.

Contractile agent	KA-OCH ₃ [μmol/l]			
	0	10	50	100
Phenylephrine	0.63 ± 0.08	0.54 ± 0.09	0.55 ± 0.07	0.64 ± 0.05
Caffeine	0.33 ± 0.04	0.40 ± 0.03	0.36 ± 0.07	0.35 ± 0.04

Values are means ± S.E.M., n = 5–7 experiments.

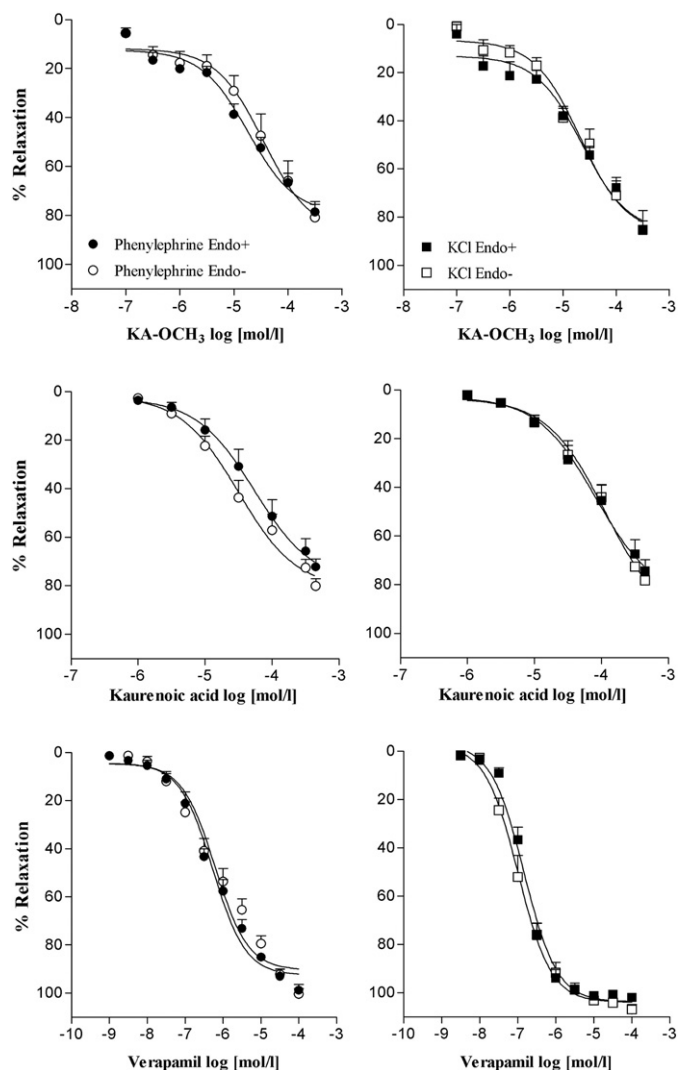


Fig. 3. Relaxation responses induced by KA-OCH₃, kaurenoic acid and verapamil on rat aortic rings. The relaxation induced by these compounds was studied on endothelium intact (Endo+) and endothelium denuded (Endo-) rat aortic rings sub-maximally pre-contracted with either phenylephrine (0.1 μmol/l, left) or KCl (30 mmol/l, right). Steady tension was evoked by phenylephrine or KCl and then KA-OCH₃ (0.1–300 μmol/l), kaurenoic acid (1–450 μmol/l) or verapamil (10 nmol/l to 100 μmol/l) were added cumulatively.

(a selective iNOS inhibitor), indomethacin (a non-selective cyclooxygenase inhibitor) and tetraethylammonium (a non-selective K⁺ channel blocker) did not have a significant effect on KA-OCH₃-induced relaxation in either endothelium-intact or endothelium-denuded rings (Table 5). The magnitude of contraction induced by phenylephrine was similar in the presence of the different inhibitors (data not shown).

3.6. Confocal microscopy and image analysis

Analysis of our data using Eq. (1) revealed that [Ca²⁺]_i was significantly increased after addition of phenylephrine or KCl to endothelial cells (Fig. 4). The increase in [Ca²⁺]_i mediated by phenylephrine or KCl was significantly inhibited by verapamil or KA-OCH₃. Similarly, [Ca²⁺]_i was significantly increased after addition of phenylephrine or KCl to smooth muscle cells (Fig. 4). The increase in [Ca²⁺]_i induced by phenylephrine and KCl in smooth muscle cells was significantly reduced by treatment with verapamil or KA-OCH₃.

Table 5

Effect of L-NAME, 7-nitroindazole, 1400 W, ODQ, wortmannin, indomethacin and tetraethylammonium on KA-OCH₃-induced relaxant responses (% relaxation) of endothelium-intact or denuded rat aortic rings pre-contracted with phenylephrine.

Groups	Endothelium-intact		Endothelium-denuded	
	E _{max}	pD ₂	E _{max}	pD ₂
Control	80.6 ± 2.8 (10)	5.29 ± 0.1	80.8 ± 6.4 (6)	5.08 ± 0.07
L-NAME 100 μmol/l	78.4 ± 6.6 (6)	4.6 ± 0.2 ^a	84.1 ± 4.1 (6)	4.5 ± 0.1 ^a
L-NAME 300 μmol/l	37.3 ± 4.7 ^a (5)	4.7 ± 0.1 ^a	38.4 3.9 ^a (6)	4.7 ± 0.1 ^a
7-nitroindazole 100 μmol/l	74.0 ± 3.4 (5)	4.6 ± 0.1 ^a	84.2 ± 5.4 (6)	4.4 ± 0.1 ^a
1400 W 1 μmol/l	88.8 ± 8.8 (6)	4.9 ± 0.2	86.5 ± 9.1 (5)	4.8 ± 0.2
ODQ 1 μmol/l	81.5 ± 2.5 (7)	4.5 ± 0.1 ^a	85.9 ± 6.7 (6)	4.6 ± 0.13 ^a
ODQ 3 μmol/l	38.2 ± 2.3 ^a (6)	4.7 ± 0.1 ^a	38.1 ± 4.1 ^a (6)	4.7 ± 0.1 ^a
Wortmannin 0.5 μmol/l	81.2 ± 7.1 (7)	4.4 ± 0.1 ^a	68.3 ± 7.7 (7)	4.5 ± 0.1 ^a
Wortmannin 1 μmol/l	72.8 ± 9.9 (6)	4.7 ± 0.2 ^a	67.8 ± 6.4 (6)	4.6 ± 0.1 ^a
Indomethacin 10 μmol/l	83.2 ± 3.5 (8)	5.1 ± 0.1	85.4 ± 4.7 (7)	5.5 ± 0.2
Tetraethylammonium 1 mmol/l	88.1 ± 4.3 (8)	4.9 ± 0.2	93.2 ± 6.3 (5)	4.9 ± 0.2

Number between parentheses indicates the number of animals. Values are means ± S.E.M.

^a Compared to control group (ANOVA followed by Newman–Keuls multiple comparison test, P < 0.05).

3.7. Blood pressure experiments

The maximal variation in mean arterial pressure and heart rate induced by KA-OCH₃ and kaurenoic acid in conscious normotensive rats is presented in Fig. 5. The basal values for the mean arterial pressure and heart rate were 98 ± 1.7 mm Hg (n = 10) and 343 ± 6.5 bpm (n = 10), respectively. A bolus injection of KA-OCH₃ or kaurenoic acid (1 to 10 mg/kg) produced a dose-dependent decrease in mean arterial pressure and an increase in heart rate in conscious normotensive rats. The mean arterial pressure values returned to basal levels within the first minute after injection of all doses of the diterpenes. KA-OCH₃ was found to be more potent than kaurenoic acid at inducing a decrease in mean arterial pressure and an increase in heart rate.

4. Discussion

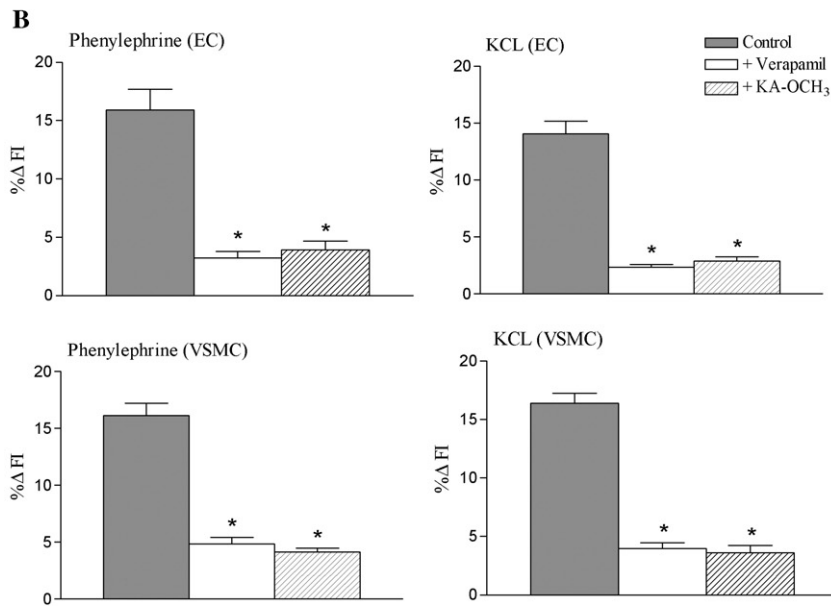
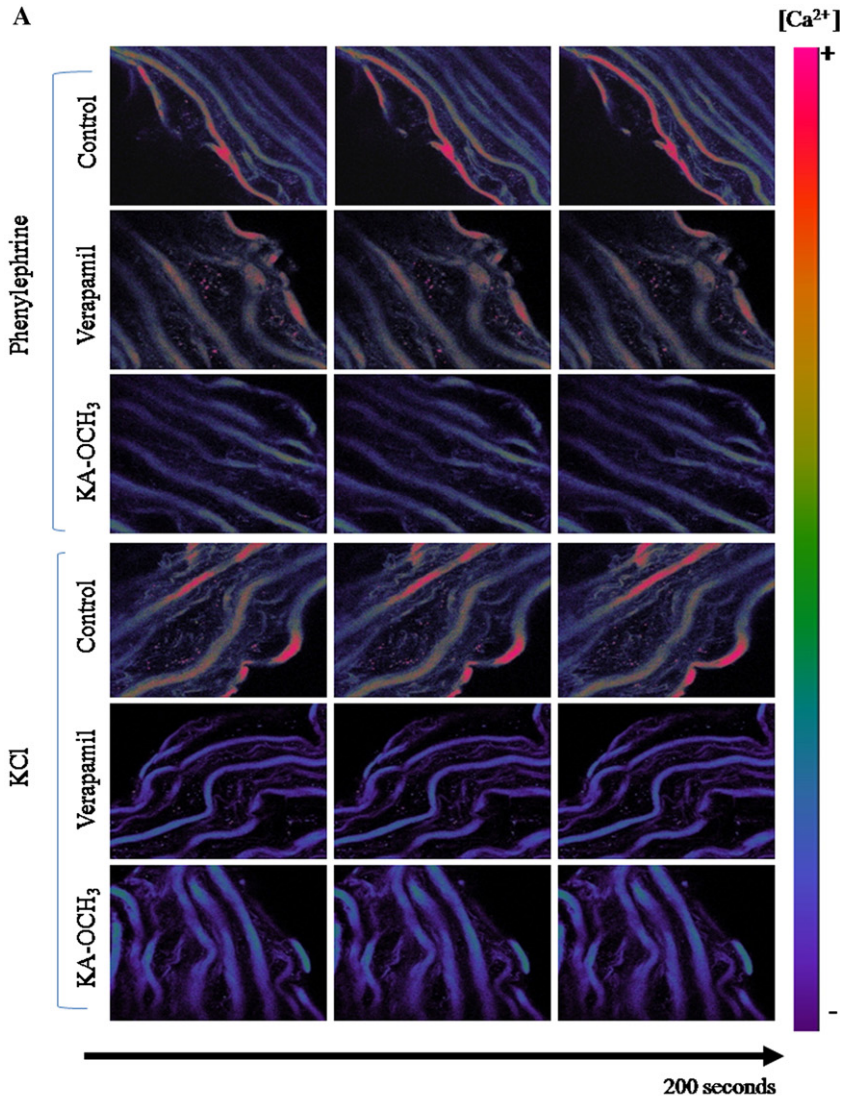
The present findings corroborate previous studies showing that alterations in the chemical structure of kauranes modify their biological actions (Ambrosio et al., 2004; Muller et al., 2003; Zamilpa et al., 2002). The carboxyl group at C-19 is an important structural element (Enriquez et al., 1984; Campos-Bedolla et al., 1997) but is not the only component responsible for the antispasmodic and relaxant effects of diterpenes (Muller et al., 2003; Ambrosio et al., 2004). In fact, Zamilpa et al. (2002) showed that *ent*-beyer-15-en-19-oic acid, a diterpene that differs slightly from kaurenoic acid in ring D, was more effective than kaurenoic acid at inhibiting electrically induced contractions in guinea-pig ileum. In the present study, we showed that KA-OCH₃, a semi-synthetic derivative diterpene that differs from kaurenoic acid by the insertion of an α-methoxy group in ring D at C-16, inhibits the vascular contraction induced by phenylephrine and KCl and induces relaxation in arteries pre-contracted with both phenylephrine and KCl. However, it is important to note that KA-OCH₃ is approximately ten times more potent than kaurenoic acid at inducing vascular relaxation. Based in such observations, we concluded that this chemical modification increased kaurenoic acid's vascular action.

Two types of stimulants are widely used in vascular smooth muscle to increase the cytosolic Ca²⁺ level: high-K⁺-induced membrane depolarisation and contractile agonists such as phenylephrine (Karaki et al., 1997). An increase in the cytosolic Ca²⁺ concentration is the major trigger for smooth muscle contraction because it leads to the formation of the Ca²⁺-calmodulin complex, inducing signalling that culminates in muscle contraction (Araujo and Bendhack, 2003). Phenylephrine-induced contraction is mediated by an increase in Ca²⁺ influx through both receptor-operated channels (Hirata et al., 1998) and voltage-sensitive channels (Wesselman et al., 1996; Lee et al., 2001), whereas KCl-induced contraction in smooth muscle is mediated by cell

membrane depolarisation and an increase in Ca²⁺ influx through voltage-operated Ca²⁺ channels (Hudgins and Weiss, 1968; Somlyo and Somlyo, 1994). The present findings show that KA-OCH₃ inhibited the vascular contraction induced by phenylephrine and KCl (30 mmol/l) suggesting that KA-OCH₃ blocks Ca²⁺ influx through interference with both voltage- and receptor-operated channels. Moreover, we observed that KA-OCH₃ induced endothelium-independent relaxation in aortic rings pre-contracted with both phenylephrine and KCl and inhibited the contraction induced by CaCl₂ in a Ca²⁺-free solution containing KCl or phenylephrine. Finally, using the selective probe Fluo-3 AM, we found that KA-OCH₃ inhibits the increase in [Ca²⁺]_i induced by phenylephrine and KCl. Taken together, our results support the notion that KA-OCH₃ acts as a Ca²⁺ channel blocker.

The effect of KA-OCH₃ on Ca²⁺ release from phenylephrine- and caffeine-sensitive intracellular stores was also analysed. In our study, the magnitude of contraction induced by phenylephrine was higher than that induced by caffeine. Phenylephrine induces the production of inositol triphosphate (IP₃), which activates IP₃ receptors (Eckert et al., 2000), whilst caffeine stimulates ryanodine receptors (Xu et al., 1998). Both receptors mediate the release of Ca²⁺ from the sarcoplasmic reticulum, an essential step in smooth muscle contraction. However, in addition to the production of IP₃, stimulation of α₁ receptors by phenylephrine results in the regulation of multiple effector systems such as the production of diacylglycerol, which in turn activates protein kinase C. The latter phosphorylates the light chain of myosin, which is associated with the development of tension (Stull et al., 1990). The activation of protein kinase C by phenylephrine could be responsible for the increase in tension induced by this agonist compared to caffeine. The lack of effect of KA-OCH₃ on Ca²⁺ release from intracellular stores sensitive to phenylephrine and caffeine suggests that the vascular effect of the diterpene does not involve a reduction in Ca²⁺ release from intracellular stores. Moreover, this result might explain the fact that KA-OCH₃ displayed similar relaxant responses in preparations pre-contracted with phenylephrine and KCl. Phenylephrine-induced contraction involves the opening of Ca²⁺ channels on the plasma membrane and the mobilisation of intracellular Ca²⁺ stores, whilst KCl-induced contraction is mediated by membrane depolarisation and the opening of Ca²⁺ channels on the membrane. Since KA-OCH₃ does not affect Ca²⁺ release from intracellular stores, this compound should act as a blocker of Ca²⁺ channels located on the plasma membrane.

Diterpenes exert their cardiovascular effects by acting at multiple sites (Melis, 1997; Tirapelli et al., 2004a,b). The NO pathway was described to be involved in the cardiovascular action of some diterpenes (De Oliveira et al., 2006; Silva et al., 2005a, 2005b). In our study, pre-treatment of endothelium-intact or endothelium-denuded rings with L-NAME was associated with a rightward displacement of



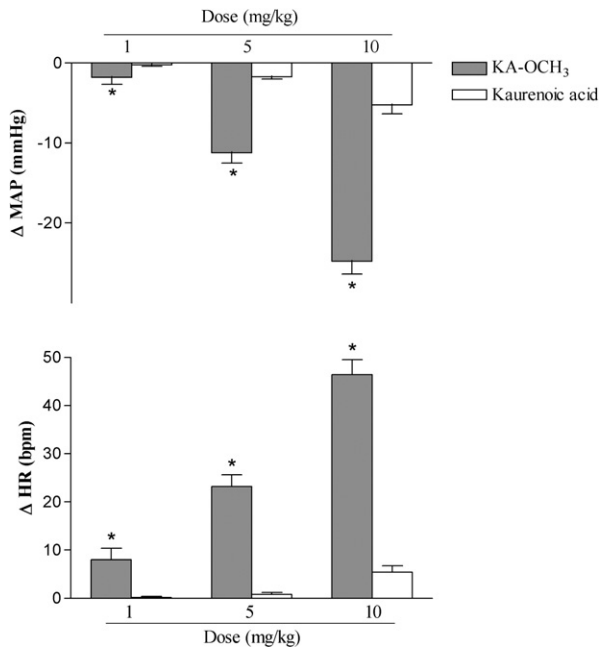


Fig. 5. Effect of KA-OCH₃ and kaurenoic acid (1–10 mg/kg) on mean arterial pressure and heart rate. Maximal variation in mean arterial pressure (mm Hg) and heart rate (bpm) induced by intravenous injection of KA-OCH₃ or kaurenoic acid was evaluated in conscious normotensive rats. Each bar represents the mean \pm S.E.M. of 5 experiments. *Compared with kaurenoic acid ($P < 0.05$, ANOVA followed by Newman–Keuls multiple comparison test).

the curve for KA-OCH₃, indicating the participation of NO in the vasorelaxant effects of KA-OCH₃. Our data suggests that KA-OCH₃ induces the generation of NO in smooth muscle, which was demonstrated to possess biochemical pathways to produce NO (Carrillo-Sepúlveda et al., 2010; Schini and Vanhoutte, 1991). As NO synthase is an enzyme that occurs in three major isoforms (endothelial, neuronal and inducible) (Förstermann et al., 1994), we investigated which of these isoforms is involved in KA-OCH₃-induced relaxation. The rightward displacement of the concentration–response curve for KA-OCH₃ in endothelium-intact and endothelium-denuded rings in the presence of 7-nitroindazole suggests that the activation of nNOS plays a role in the vasorelaxant effects of KA-OCH₃. nNOS is expressed in vascular smooth muscle cells (Carrillo-Sepúlveda et al., 2010), and the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway has been shown to upregulate the phosphorylation of nNOS, activating the enzyme and promoting vasodilatation (Carrillo-Sepúlveda et al., 2010; El-Mas et al., 2009). Taken together, our results suggest that the vasodilator effect of KA-OCH₃ is partly mediated by increased production of NO, which occurs rapidly upon activation of PI3K/Akt signalling, as suggested by the ability of the PI3K inhibitor wortmannin to alter KA-OCH₃-induced relaxation. Since NO induces relaxation in vascular smooth muscle through a cGMP-dependent mechanism (Moncada et al., 1989), we sought to determine a possible requirement for the cGMP pathway in the vasorelaxant action of KA-OCH₃. A selective inhibitor of guanylyl cyclase enzyme, ODC (Garthwaite et al., 1995), inhibited the vasorelaxant action of KA-OCH₃; this finding confirms the involvement of the NO-cGMP pathway in KA-OCH₃-mediated vasorelaxant responses.

Vasodilator cyclooxygenase product(s) of non-endothelial origin have been described to induce endothelium-independent relaxation (Cherry et al., 1982; Förstermann et al., 1986) and to mediate the relaxation induced by diterpenes (De Oliveira et al., 2006; Tirapelli et al., 2004b). Indomethacin, a non-selective cyclooxygenase inhibitor, was not found to affect the relaxation caused by KA-OCH₃. Therefore, it appears that cyclooxygenase pathways do not play an appreciable role in mediating KA-OCH₃'s vasodilatory effects.

The opening of K⁺ channels in the cell membranes of smooth muscle cells in arteries increases K⁺ efflux, causing membrane potential hyperpolarisation, which leads to vasodilatation (Nelson and Quayle, 1995). Tetraethylammonium, a non-selective blocker of K⁺ channels, did not alter the relaxation induced by KA-OCH₃, ruling out the participation of K⁺ channels in the vasorelaxant action induced by this diterpene. Interestingly, we previously demonstrated that the vasorelaxation induced by kaurenoic acid involves the opening of K⁺ channels (Tirapelli et al., 2004a). Based on these findings, we suggest that the chemical modification of kaurenoic acid to obtain KA-OCH₃ not only increased the potency of the compound but also altered its mechanism of action. Thus, the present findings highlight the great importance of studying other diterpenes in a structure–activity context to determine which other factors contribute to the antispasmodic and relaxant activity displayed by this class of compounds.

Several reports show that kaurane-type diterpenes induce hypotension in conscious normotensive rats by a mechanism that involves blockade of extracellular Ca²⁺ influx in the vascular smooth muscle (Somova et al., 2001; Tirapelli et al., 2008a), further indicating that the hypotensive action displayed by natural occurring diterpenes is related to their myorelaxant activity. Since KA-OCH₃ induces vascular relaxation, we hypothesised that this diterpene could exert a hypotensive action. Bolus injection of KA-OCH₃ produced a dose-dependent decrease in mean arterial pressure in conscious normotensive rats. Kaurenoic acid induced a less pronounced decrease in blood pressure compared to KA-OCH₃, supporting the idea that this chemical modification of kaurenoic acid increased its hypotensive action. The experiments performed on isolated vascular tissues suggest that KA-OCH₃-induced hypotension is mediated by the direct vasorelaxant action of this diterpene on the vascular smooth muscle, a response that involves the blockade of extracellular Ca²⁺ influx. Clinically, Ca²⁺ antagonists are used for the treatment of hypertension due to their ability to induce smooth muscle relaxation (Sahney, 2006). Thus, based on its vascular effects, we suggest that KA-OCH₃ could potentially exert antihypertensive effects in vivo.

In contrast, KA-OCH₃ induced an increase in heart rate. Previously, the diterpene 14-deoxy-11,12-didehydroandrographolide was found to cause a significant decrease in heart rate in normotensive rats (Zhang et al., 1998). In isolated rat right atria, this diterpene antagonises the positive chronotropic effect elicited by the β -adrenoreceptor agonist isoproterenol, which supports the idea that the bradycardic effect elicited by 14-deoxy-11,12-didehydroandrographolide in vivo is due to direct β_1 -adrenoreceptor blockade. In our study, the increase in heart rate induced by KA-OCH₃ was probably due to reflex tachycardia. However, further studies in isolated rat atria should be performed to verify whether KA-OCH₃ displays a direct effect on heart contractility.

The present study, using a combined in vivo and in vitro approach, shows that KA-OCH₃ induces vascular relaxation and hypotension. The mechanisms underlying the cardiovascular actions of KA-OCH₃ involve two distinct actions, 1) blockade of extracellular Ca²⁺ influx and 2) activation of the NO-cGMP pathway. Finally, we conclude that

Fig. 4. Effect of phenylephrine and KCl on transient [Ca²⁺]_i in the presence of KA-OCH₃ or verapamil: slices of aortic rings with endothelium-intact were preloaded with Fluo-3 AM and then stimulated with phenylephrine (10 μ M) or KCl (60 mmol/l) in the absence (control) or presence of KA-OCH₃ (100 μ M) and verapamil (1 μ M). (A) Serial Ca²⁺ images of Fluo-3 AM fluorescence in slices of rat aorta were recorded at the times (t) 0, 3, and 4.5 s and KCl or phenylephrine were added at 1.5 s. (B) Histogram shows the mean changes of fluorescence intensity (% Δ FI) of [Ca²⁺]_i in vascular smooth muscle cells (VSMC) and endothelial cells (EC) from aortic rings preloaded with Fluo-3 AM after addition of phenylephrine or KCl or the combination of these stimulants with verapamil and KA-OCH₃. Each bar represents the mean \pm S.E.M. of 4 experiments. *Compared with respective control group ($P < 0.05$, ANOVA followed by Newman–Keuls multiple comparison test).

structural alterations can improve the cardiovascular activity of kaurane-type diterpenes and alter their mechanism of action.

Conflict of interest

The authors of the present manuscript declare that there are no financial links including consultancies with manufacturers of material or devices described in the paper as well as links to the pharmaceutical industry or regulatory agencies or any other potential conflicts of interest.

Acknowledgements

We thank Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) for funds and grants (process number 2009/52629-4).

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