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Role of the carboxylic group in the antispasmodic and vasorelaxant action displayed by kaurenoic acid

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Abstract

The present work describes the investigation of the role of the carboxylic group in the structure–activity relationship of the diterpene *ent*-kaur-16-en-19-oic acid (kaurenoic acid, KA) in inhibiting rat aorta contraction. For this purpose the methylation of the C-19 carboxyl group of KA was carried out. The effects of the obtained *ent*-methyl-kaur-16-en-19-oate (KAMe) were compared with those induced by KA. Vascular reactivity experiments showed that KA (50 and 100 μM) concentration-dependently inhibited KCl-induced contraction in both endothelium-intact and denuded rat aortic rings. On the other hand, KAMe attenuated KCl-induced contraction at 100 μM, but not at 50 μM. KA also reduced $CaCl_2$-induced contraction in Ca^{2+}-free solution containing KCl (30 mM). Again, KAMe produced a less accentuated reduction in $CaCl_2$-induced contraction than that induced by the acid KA. KAMe (1–450 μM) concentration-dependently relaxed KCl-pre-contracted rings (percentages of relaxation 82.57 ± 1.65 and 70.55 ± 4.71, respectively) with denuded endothelium. Similarly, the relaxation induced by KA on phenylephrine (Phe)-pre-contracted rings ($73.06 \pm 3.68\%$) was more pronounced than that found for KAMe ($53.68 \pm 4.75\%$). Pre-incubation of denuded rings for different periods with KA and KAMe showed that the equilibrium periods required by each compound to achieve its maximal inhibitory response on KCl-induced contraction are different. Collectively, our results provide functional evidence that methylation of the C-19 carboxyl group of KA reduces but does not abolish the antispasmodic activity displayed by KA. Additionally, we showed that the equilibrium period is a critical step for the inhibitory effect displayed by kaurane-type diterpenes.

Introduction

Many biological assays have provided evidence that kaurane-type diterpenes exert antispasmodic and relaxant actions on smooth muscle. We have previously demonstrated that the diterpene *ent*-kaur-16-en-19-oic acid (kaurenoic acid, KA) exhibits antispasmodic action on vascular smooth muscle. The diterpene inhibits the contraction induced by KCl and phenylephrine (Phe) in the isolated rat carotid artery with intact endothelium (Da Costa et al 2000; Tirapelli et al 2002). Additionally, we found that KA relaxes segments of endothelium-intact rat carotid pre-contracted with either Phe or KCl (Tirapelli et al 2003). More recently, we have provided evidence that the antispasmodic and relaxant effects elicited by KA on vascular smooth muscle involve blocked extracellular Ca^{2+} influx (Tirapelli et al 2004).

Previously, Bejar et al (1984) described that kauradienoic acid relaxed uterine strips pre-contracted with Ca^{2+}, suggesting that the diterpene could reduce extracellular Ca^{2+} influx. Kauradienoic acid has also been described as displaying inhibitory action on the spontaneous contractility of rat, guinea pig and human uteri (Enríquez et al 1984). These studies assumed that the carboxylic group at C-19 is a prerequisite for the inhibitory activity of kauradienoic acid on extracellular Ca^{2+} influx. Likewise, Campos-Bedolla et al (1997) concluded that the carboxylic group plays an important role in the structure–activity relationship of the kauranes as the 16α-hydroxy-*ent*-kauran-19-oic acid methyl ester displayed an inhibitory effect that was significantly less potent than its respective acid at inhibiting serotonin-induced contraction. On the other hand, our recent data demonstrate that two kauranes, which lack the C-19

carboxylic group, also reduce the contraction of rat aorta induced by Phe (Muller et al 2003).

These contradictory findings prompted us to investigate the role of the C-19 carboxylic group in the structure–activity relationship of KA in inhibiting vascular smooth muscle contraction. With this purpose in mind, a chemical alteration of that group was carried out and the obtained methyl ester derivative (KAMe) was tested for its activity.

Material and Methods

Isolation of KA

Individuals of *Viguiera robusta* Gardn. (Asteraceae) were collected by F. B. Da Costa and authenticated by J. N. Nakajima (Universidade Federal de Uberlândia, Brazil) and E. E. Schilling (University of Tennessee, USA). Voucher specimens (reg. number FBC # 60) are deposited in the herbarium SPFR of the Departamento de Biologia, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil. Air-dried (40°C) powdered roots (3 g) were macerated with CH_2Cl_2 (150 mL) in a sonicator at room temperature for 15 min. After solvent evaporation under vacuum the crude extract (550 mg) was analysed by TLC and IR spectroscopy. According to previous work (Da Costa et al 1996a, b) this preliminary analysis indicated the presence of diterpene acids.

The crude extract (500 mg) was submitted to medium-pressure chromatography (silica gel, Merck 9385, 40–63 μM; N_2 as flow gas, isocratic; *n*-hexane:EtOAc 7:3) to yield 35 fractions (15 mL each), which were combined into six after TLC analysis. Fraction 3 (100 mg) furnished 20 mg of pure KA, which was isolated by preparative TLC (silica gel, Merck; *n*-hexane:EtOAc 4:1) and identified by means of spectrometric analysis (NMR) by comparison with an authentic sample and data from the literature (Da Costa et al 1996a, b).

About 200 mg of KA, previously isolated in our laboratory from *Viguiera aspillioides* Gardn. (Asteraceae), was treated with CH_2N_2 in Et_2O (Da Costa et al 1996a), yielding the respective methyl ester derivate (KAMe), which was identified by means of spectrometric analysis and data from the literature (Da Costa et al 1996a, b).

The purity (95–98%) of KA (colourless prisms, mp 129–130°C, $[\alpha]_D^{25} = -110°$, $c = 1.5$, $CHCl_3$) and KAMe (white leaflets, mp 72–75°C, $[\alpha]_D^{25} = -91.9°$, $c = 7.93$, $CHCl_3$) was estimated by TLC analysis using different solvent systems as well as ^1H and ^{13}C NMR spectral data analysis.

Vessel ring preparation

Male Wistar rats weighing between 200 and 250 g (50–60 days old) were anaesthetized and killed by aortic exsanguination in accordance with the Ethical Animal Committee from 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 (Letica Scientific Instruments) to measure tension in the vessels. The rings were placed in a 5-mL organ chamber containing Krebs solution gassed with 95%O_2/5%CO_2, and maintained at 37°C. The composition of Krebs solution was as follows (mM): NaCl 118.0; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 15.0; glucose 5.5; $CaCl_2$ 2.5. The rings were stretched to an optimal basal tension of 1.0 g, which was determined by length–tension relationship experiments, and then allowed to equilibrate for 60 min, with the bath fluid being changed every 15–20 min. In some rings, the endothelium was removed mechanically by gently rolling the lumen of the vessel on a thin wire. Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (1 μM) in the presence of contractile tone induced by Phe (0.1 μM). For studies of endothelium-intact vessels, the ring was discarded if relaxation with acetylcholine was not 80% or greater. For studies of endothelium-denuded vessels, the rings were discarded if there was any degree of relaxation.

Effect of KA and KAMe on contractions induced by KCl and $CaCl_2$

After equilibration, cumulative concentration–response curves for KCl (10–90 mM) were determined. The curves were obtained in intact and denuded rings by a stepwise increase in the concentration of KCl. Additions were made as soon as a steady response was obtained from the preceding concentration. The curves for KCl were determined in the absence of KA or KAMe (control group) or after a 60-min incubation period with KA or KAMe (50 or 100 μM) (Tirapelli et al 2004).

To assess the effects of KA and KAMe on $CaCl_2$-induced contractions, endothelium-denuded rings were first contracted with Phe (0.1 μM) to deplete the intracellular Ca^{2+} stores in Ca^{2+}-free solution (approximately 90 min) containing EGTA (1 mM) and then rinsed in Ca^{2+}-free solution (without EGTA) containing KCl (30 mM). The cumulative concentration–response curves for $CaCl_2$ (0.05–2 mM) were obtained in the absence of KA or KAMe (control group) or after a 60-min incubation period with KA or KAMe (50 or 100 μM) (Tirapelli et al 2004).

Effect of KA, KAMe and nifedipine on aortic rings contracted with Phe or KCl

In another set of experiments, steady tension was evoked by Phe 0.03 μM or KCl 30 mM (to induce contraction of similar magnitude) and then KA or KAMe was added cumulatively (1–450 μM). The effects of KA and KAMe on attenuating Phe- or KCl-induced sustained contraction were evaluated in endothelium-denuded rings. For comparison, the effect of nifedipine (10^{-8}–10^{-6} M), a blocker of voltage-dependent Ca^{2+} channels, was also evaluated against the contractions induced by Phe and KCl in endothelium-denuded rings. Relaxation was expressed as percentage change from the KCl- or Phe-contracted levels.

Effect of the period of incubation on the inhibitory action induced by KA and KAMe

Firstly, the aortic rings were contracted with KCl 30 mM (control). Subsequently, the preparations were washed out and pre-incubated with KA (50 or 100 μM) for 30, 60 or 90 min and with KAMe (50 or 100 μM) for 30, 60, 90 or 120 min. After these periods of incubation, a new stimulation was performed with KCl. The effect of the calcium channel blocker nifedipine (1 μM, 30 and 60 min) was also analysed.

The stimulation with KCl was determined on the same ring, so that each ring served as its own control. The first stimulation determined before the pre-incubation with the diterpenes was considered as the control. Vessel rings from the same animal that were not exposed to the diterpenes or nifedipine served as time controls.

Drugs

The following drugs were used: phenylephrine hydrochloride, acetylcholine hydrochloride, nifedipine (Sigma, St Louis, MO), potassium chloride and calcium chloride (Synth, São Paulo, Brazil). Nifedipine, KA and KAMe were prepared as stock solutions in ethanol. The other drugs were dissolved in distilled water. The bath concentration of ethanol 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.

Statistical analysis

To study the effect of KA and KAMe on attenuating contraction or inducing relaxation, two pharmacological parameters were analysed: E_{max} (maximal effect generated by the agonist) and pD_2 (−log EC50). Results were expressed as means ± s.e.m. Statistical analysis of the E_{max} values was performed using one-way analysis of variance (ANOVA). The same analysis was applied to pD_2 values. Post-hoc comparisons were performed after ANOVA analysis using the Bonferroni multiple comparison test. The significance level considered in all tests was 0.05.

Results

Effect of KA and KAMe on contractions induced by KCl and $CaCl_2$

The chemical structures of KA and KAMe are represented in Figure 1. The E_{max} values for KCl in endothelium-intact or denuded rings were depressed in the presence of KA at 50 and 100 μM (Figure 2, Table 1A; $P < 0.05$). At these concentrations, the diterpene reduced the pD_2 values for KCl in either endothelium-intact or denuded rings (Figure 2, Table 1B; $P < 0.05$). On the other hand, KAMe at 100 μM, but not at 50 μM, significantly attenuated the E_{max} values for KCl in endothelium-intact or denuded rings (Figure 2, Table 1A; $P < 0.05$).

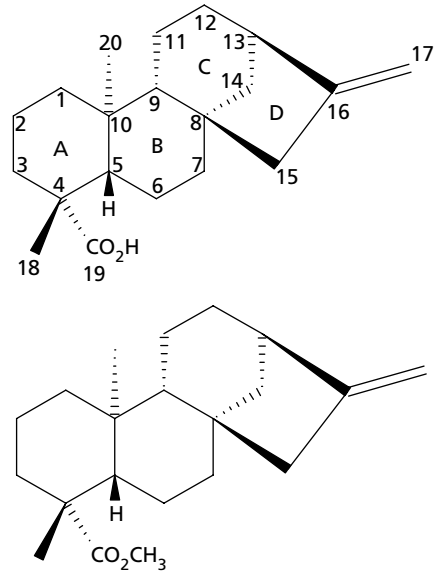

Figure 1 Chemical structures of kaurenoic acid (KA) and kaurenoic acid methyl ester (KAMe).

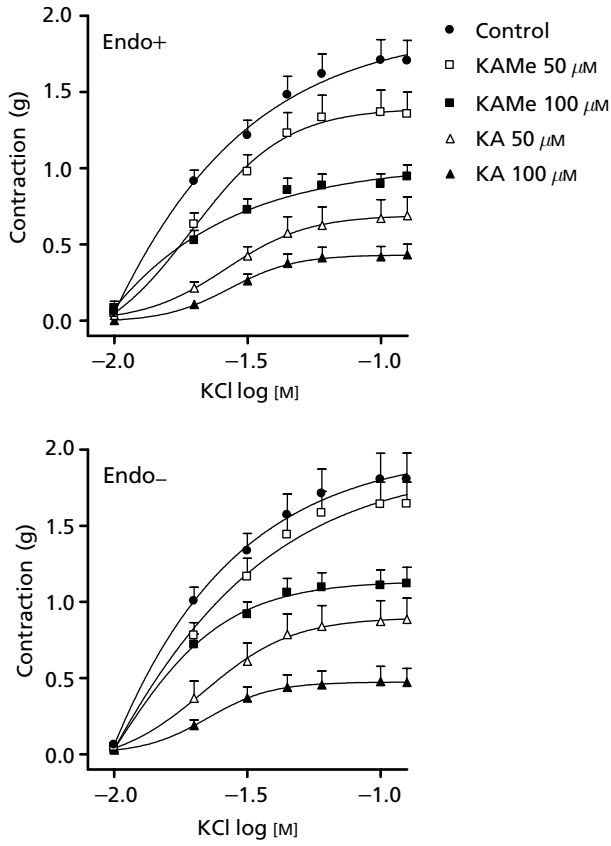

Figure 2 Effect of KA and KAMe on KCl-induced contractile response in rat aortic rings. Concentration–response curves for KCl were determined in endothelium-intact (Endo+) or endothelium-denuded (Endo−) rings. The curves were determined in the absence of KA or KAMe (control) or after a 60 min period of incubation with KA or KAMe (50 or 100 μM).

Table 1 Effect of KA and KAMe on the E_{max} (g) (A) and pD_2 values (B) for KCl in endothelium-intact (Endo+) or denuded (Endo−) aortic rings

	Control	KA (μM)		KAMe (μM)	
		50	100	50	100
A					
Endo+	1.71 ± 0.13	0.69 ± 0.12^{a}	$0.44 \pm 0.07^{a,b}$	$1.40 \pm 0.14^{b,c}$	$0.95 \pm 0.08^{a,b,c}$
Endo−	1.80 ± 0.17	0.88 ± 0.13^{a}	$0.47 \pm 0.09^{a,b}$	$1.64 \pm 0.14^{b,c}$	$1.12 \pm 0.11^{a,b,c}$
B					
Endo+	1.72 ± 0.15	1.50 ± 0.07^{a}	1.55 ± 0.09^{a}	1.63 ± 0.09	1.66 ± 0.09
Endo−	1.75 ± 0.17	1.53 ± 0.06^{a}	1.56 ± 0.03^{a}	1.71 ± 0.06	1.69 ± 0.11

Values are mean ± s.e.m. of n = 7–10 experiments. [a]Compared to control group. [b]Compared to KA 50 μM. [c]Compared to KA 100 μM. ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$.

KAMe did not alter the pD_2 values for KCl in either intact or denuded rigs (Figure 2, Table 1B).

As can be seen in Figure 3, pre-treatment with KA and KAMe attenuated $CaCl_2$-induced contraction of denuded rat aorta exposed to Ca^{2+}-free medium containing KCl.

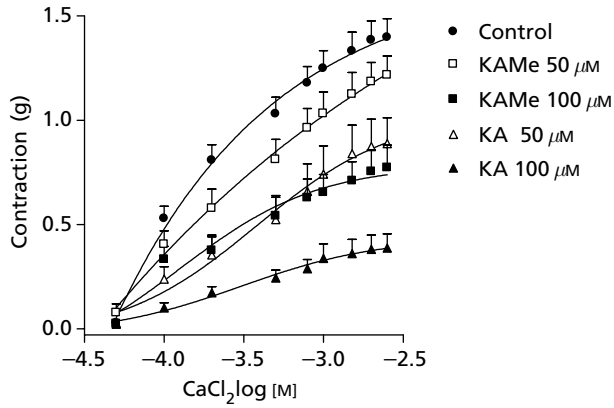

Figure 3 Effect of KA and KAMe on $CaCl_2$-induced contractile response in endothelium-denuded aortic rings. Concentration–response curves for $CaCl_2$ were determined in Ca^{2+}-free solution containing KCl (30 mM). The curves were determined in the absence of KA or KAMe (control) or after a 60 min period of incubation with KA or KAMe (50 or 100 μM).

$CaCl_2$ induced a concentration-dependent contraction of rat aortic rings. Pre-incubation of the rings with KA at 50 or 100 μM significantly reduced the E_{max} values for $CaCl_2$ (ANOVA, $P < 0.05$). Likewise, KA at these concentrations produced a rightward displacement of the concentration–response curve for KCl. On the other hand, KAMe at 50 μM did not alter either the E_{max} or pD_2 values for $CaCl_2$. Conversely, at 100 μM the methyl ester significantly attenuated $CaCl_2$-induced contraction. However, the inhibitory effect induced by KAMe at 100 μM was less pronounced than that found for KA at the same concentration (Table 2, $P < 0.05$).

Effect of KA, KAMe and nifedipine on aortic rings pre-contracted with Phe or KCl

KA and KAMe at concentrations ranging from 1 to 450 μM significantly inhibited the sustained tonic contraction induced by Phe and KCl in a concentration-dependent manner (Figure 4). The E_{max} values (percentage of relaxation) of the relaxant effect of KA for denuded rings pre-contracted with KCl were significantly different from those obtained for KAMe ($P < 0.05$). Similarly, KA also exerted a more pronounced relaxation in Phe-pre-contracted aortic rings when compared with KAMe ($P < 0.05$). In the arteries pre-contracted with either KCl or Phe there was no difference between the pD_2 values

Table 2 Effect of KA and KAMe on the E_{max} (g) and pD_2 values for $CaCl_2$ in endothelium-denuded aortic rings

	Control	KA (μM)		KAMe (μM)	
		50	100	50	100
E_{max}	1.39 ± 0.09	$0.89 \pm 0.12^{a,b,c}$	$0.38 \pm 0.07^{a,c}$	1.22 ± 0.09^{b}	$0.77 \pm 0.10^{a,b,c}$
pD_2	3.83 ± 0.27	$3.30 \pm 0.18^{a,c}$	$3.44 \pm 0.12^{a,c}$	3.75 ± 0.19	$3.53 \pm 0.14^{a,c}$

Values are mean ± s.e.m., n = 6–10 experiments. [a]Compared to control group. [b]Compared to KA 100 μM. [c]Compared to KAMe 50 μM. ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$.

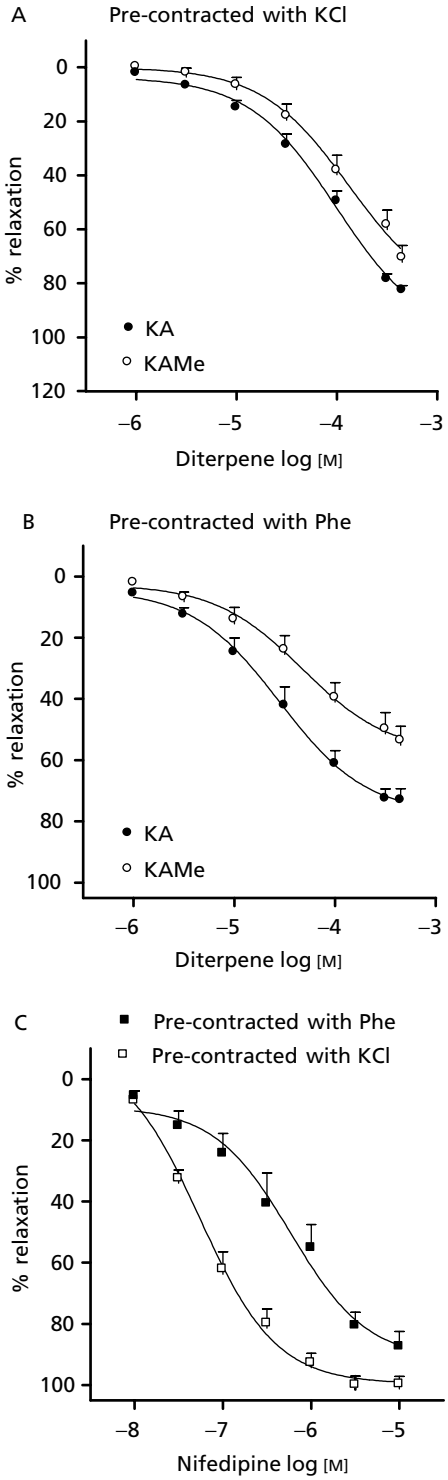

Figure 4 Relaxation responses induced by KA, KAMe (A, B) and nifedipine (C) on rat aortic rings. The relaxation induced by the three compounds were studied on endothelium-denuded rat aortic rings submaximally pre-contracted with either Phe or KCl. Steady tension was evoked by Phe or KCl and then KA, KAMe (1–450 μM) or nifedipine (10^{-8}–10^{-6} M) were added cumulatively.

for KA and KAMe. The relaxation induced by nifedipine in denuded rings pre-contracted with Phe was significantly different from that found for KA and KAMe ($P < 0.05$). Also, the pD_2 values found for nifedipine were higher than those found for the diterpenes (Table 3, $P < 0.05$).

Effect of the period of incubation on the inhibitory action induced by KA and KAMe

No differences in the E_{max} values induced by KCl (30 mM) in time-control experiments were detected (data not shown). Pre-treatment of the rings with KA and KAMe inhibited the contraction induced by a single concentration of KCl (30 mM) in a time- and concentration-dependent manner. At 50 μM both compounds achieved their maximal inhibitory activity after a pre-incubation period of 60 min. However, KA displayed a more pronounced inhibition of KCl-induced contraction than that found for KAMe ($P < 0.05$). Exposure of aortic rings to KA and KAMe at 100 μM inhibited KCl-induced contraction with a maximum after incubation for 60 and 90 min, respectively. Again, the percentage inhibition induced by KA was more pronounced than that found for KAMe ($P < 0.05$). The maximal inhibition induced by nifedipine was observed after 30 min of incubation. The inhibitory effect exerted by nifedipine was more accentuated than that produced by the diterpenes (Table 4, $P < 0.05$).

Discussion

The present findings corroborate those of our previous study conducted in rat isolated aorta, namely that KA displays antispasmodic and relaxant effects (Tirapelli et al 2004). Moreover, we have provided evidence that the methyl ester derivative (KAMe) obtained from KA, in which a chemical alteration of the carboxylic group at C-19 was performed, also exhibits antispasmodic and relaxant actions.

Our data show that KA, as well as KAMe, concentration-dependently reduces the contractions induced by KCl in endothelium-intact and denuded aortic rings. Since the contractions of rat aortic rings induced by KCl rely almost exclusively on Ca^{2+} influx through activation of voltage-sensitive channels (Hudgins & Weiss 1968), it can be suggested that both compounds can block extracellular Ca^{2+} influx through Ca^{2+} channels presented in the vascular smooth muscle cells. However, further experiments should be performed to clarify this point. It is important to note that the inhibitory effect displayed by KA is more pronounced than that found for KAMe. Such a finding indicates that the structural modification reduces but does not abolish the inhibitory activity displayed by KA.

In the present study we observed that in the presence of KA and KAMe there was a rightward shift in the concentration–response curves for $CaCl_2$ with a decrease in the E_{max} values. However, we found that KAMe at 100 μM produced a less accentuated reduction in the E_{max} value for $CaCl_2$ than that induced by KA at the same concentration.

Table 3 Effect (% relaxation) of KA, KAMe and nifedipine on aortic rings pre-contracted with Phe or KCl

	KA		KAMe		Nifedipine	
	E_{max}	pD_2	E_{max}	pD_2	E_{max}	pD_2
KCl	82.57 ± 1.65	3.93 ± 0.12	70.55 ± 4.71^{a}	3.77 ± 0.14	$99.87 \pm 2.74^{a,b,c}$	$7.14 \pm 0.25^{a,c}$
Phe	73.06 ± 3.68^{a}	4.54 ± 0.12	$53.68 \pm 4.75^{a,b}$	4.10 ± 0.11	$87.50 \pm 5.04^{b,d}$	$6.30 \pm 0.17^{b,d}$

Values are mean ± s.e.m., n = 9–14 experiments. [a]Compared to KA (pre-contracted with KCl). [b]Compared to KA (pre-contracted with Phe). [c]Compared to KAMe (pre-contracted with KCl). [d]Compared to KAMe (pre-contracted with Phe). ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$.

Table 4 Percentage inhibition induced by KA and KAMe on KCl-induced contraction of isolated rat aorta after different incubation periods

Min	KA		KAMe		Nifedipine
	50 μM	100 μM	50 μM	100 μM	1 μM
30	12.82 ± 4.94^{a}	$72.75 \pm 4.33^{a,e}$	15.98 ± 2.20^{a}	34.05 ± 5.55^{a}	83.76 ± 2.40^{a}
60	$48.91 \pm 6.72^{a,b,d}$	$70.85 \pm 5.03^{a,e}$	22.46 ± 4.00^{a}	33.12 ± 7.44^{a}	78.07 ± 2.55^{a}
90	$54.57 \pm 5.15^{a,b,d}$	$79.48 \pm 3.87^{a,e}$	$25.45 \pm 3.44^{a,b}$	$65.55 \pm 7.33^{a,c}$	–
120	–	–	$25.91 \pm 5.05^{a,b}$	$62.15 \pm 6.16^{a,c}$	–

Values are mean ± s.e.m., n = 6–8 experiments. The rings were initially stimulated with 30 mM KCl (control group, 100% contraction) and a second stimulation was performed after incubation with two concentrations of KA, KAMe or nifedipine. [a]Significant difference from respective control (0% inhibition). [b]Significant difference from pre-incubation for 30 min at 50 μM. [c]Significant difference from pre-incubation for 30 min at 100 μM. [d]Significant difference from respective period of incubation at 50 μM. [e]Significant difference from respective period of incubation at 100 μM. ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$.

Furthermore, KA at 50 μM attenuated $CaCl_2$-induced contraction while KAMe did not exert inhibitory action at this concentration. These results support the notion that the methylation of KA at C-19 reduces but does not abolish the ability of the diterpene to inhibit $CaCl_2$-induced contraction.

In addition to their antispasmodic activity, the diterpenes relaxed rings pre-contracted with either Phe or KCl. The pD_2 values as well as the percentage of relaxation induced by nifedipine, a well-known Ca^{2+} channel blocker, on denuded rings pre-contracted with Phe or KCl were higher when compared to those found for KA and KAMe. Thus, it can be concluded that nifedipine is more potent than these compounds at inhibiting Phe and KCl pre-contracted rings. Although we have found that KA and KAMe relaxed aortic rings with similar pD_2 values (same potency), it is important to note that the time taken for these compounds to induce their maximal relaxation was different. KA exerted its maximal relaxation in approximately 50 min while KAMe took approximately 90 min to completely achieve its maximal effect (data not shown). These results lead us to hypothesize that the equilibrium period required for each compound to achieve its maximal response is different.

To further analyse this hypothesis, the effect of the period of incubation on the inhibitory action induced by KA and KAMe was studied. The maximal inhibition induced by these kaurane-type diterpenes at 50 μM on KCl-induced contraction was observed after 60 min of incubation. However, KA induced a more pronounced inhibition than that found for KAMe. The higher concentration of KA (100 μM) achieved maximal inhibition after 30 min of incubation, while at this concentration KAMe displayed its maximal inhibition after 90 min. Again, the inhibitory activity displayed by KA was more pronounced than that found for KAMe. These results clearly show that the equilibrium period required for each compound to achieve its maximal response is different. These findings could also explain the lack of difference in the pD_2 values for the relaxant curves obtained for these kauranes. Such findings corroborate those of a previous study conducted in rat isolated aorta, namely that kauranes, which lack the C-19 carboxylic group, also reduce the contraction of rat aorta in a time-dependent manner (Muller et al 2003). Taken together, these data support our initial hypothesis that the C-19 carboxylic group takes part in the biological activity displayed by KA but it is not a prerequisite for this activity.

The present results contradict previous data in which it was assumed that the C-19 carboxylic group is essential for the antispasmodic activity displayed by kaurane-type diterpenes (Bejar et al 1984; Enríquez et al 1984). Some possibilities could be raised to explain these differences. Firstly, equilibrium conditions between the kauranes and their target may not be attained. Previously we have provided evidence that the equilibrium

period plays a crucial role in the antispasmodic effect elicited by kaurane-type diterpenes (Muller et al 2003). In such a study it was observed that two kauranes, which lack the C-19 carboxylic group, reduced the contraction of rat aorta, but a longer incubation time was necessary for these compounds to achieve their maximal response. Campos-Bedolla et al (1997) found that the ester 16α-hydroxy-*ent*-kauran-19-oic acid methyl ester, which lacks the C-19 carboxylic group, produced an inhibitory effect on serotonin-induced uterine contractions that was significantly less potent than its respective acid. The lack of effect observed by the authors could be related to the brief period of incubation (20 min) of the tissues with this compound. Secondly, the lack of effect previously observed after the C-19 carboxylic group was altered on uterine extracellular Ca^{2+} influx (Bejar et al 1984; Enríquez et al 1984) could be due to a variation in the characteristics or a contribution of the Ca^{2+} channels in different tissues.

Recently it was demonstrated that the inhibitory effect displayed by the diterpene *ent*-beyer-15-en-19-oic acid on electrically induced contractions of guinea-pig ileum is more pronounced than that found for KA (Zamilpa et al 2002). The structure of this compound is slightly different to that of KA because of some differences in ring D, such as the relative stereochemistry, the presence of a double bond between C-15 and C-16, a methyl group attached to C-13 and the lack of the exocyclic methylene group at C-16. Nevertheless, the other three rings (A, B and C) of both structures are identical. Such findings bring a new insight to the study of the structure–activity relationship of the kauranes.

Recently, it was demonstrated that the antihypertensive action of stevioside, a diterpene glycoside isolated from the leaves of *Stevia rebaudiana* (Asteraceae), is related to its ability to reduce the extracellular Ca^{2+} influx (Lee et al 2001). Furthermore, it must be pointed out that the aglycone of stevioside has the same structure as KA, with sugar moieties attached to an oxygen atom at C-13 and to the carboxyl group at C-19. Thus, the therapeutic effect of both compounds may be supposed to be similar. It is possible to suggest, considering its vascular effects, that KA is a potential agent that could exert antihypertensive action in vivo. In this case, it is important to understand the structure–activity relationship of this compound since structural alterations can improve its biological activity. The study of other modifications in the carboxylic group at C-19 could therefore be important for the clarification of the participation of this group in the antispasmodic and vasorelaxant activity of KA.

Conclusion

From our data it is possible to conclude that structural modification of the C-19 carboxylic group reduces but does not abolish the antispasmodic activity displayed by KA. In addition, we have provided evidence that the equilibrium period is a critical step for the inhibitory effect displayed by kaurane-type diterpenes.

References

Bejar, E., Lozoya, X., Enriquez, R. G., Escobar, L. (1984) Comparative effect of zoapatle (*Montanoa tomentosa*) products and of verapamil on the in vitro uterine contractility of rat. *Arch. Invest. Méd.* **15**: 223–235

Campos-Bedolla, P., Campos, M. G., Valencia-Sanches, A., Ponce-Monter, H. (1997) Effect of kauranes from *Montanoa spp* on rat uterus. *Phytother. Res.* **11**: 11–16

Da Costa, F. B., Albuquerque, S., Vichnewski, W. (1996a) Diterpenes and synthetic derivatives from *Viguiera aspillioides* with tripanomicidal activity. *Planta Med.* **62**: 577–579

Da Costa, F. B., Vichnewski, W., Herz, W. (1996b) Constituents of *Viguiera aspillioides* and *V. robusta*. *Biochem. Syst. Ecol.* **24**: 585–587

Da Costa, F. B., Ambrosio, S. R., Tirapelli, C. R., de Oliveira, A. M. (2000) Inhibitory effect of kaurenoic acid isolated from *Viguiera robusta* (Asteraceae) on the phenylephrine and KCl spasmogenic effect in rat carotid muscle. *Phytomedicine* **7** (suppl.): 77

Enriquez, R. G., Bejar, E., Lozoya, X. (1984) Role of kauradienoic acid and its methyl ester on the effect elicited by *Montanoa tomentosa* upon the uterine contractility in vitro. *Arch. Invest. Méd.* **15**: 236–238

Hudgins, A., Weiss, G. (1968) Effects of Ca^{+2} removal upon vascular smooth muscle contraction induced by norepinephrine, histamine and potassium. *J. Pharm. Exp. Ther.* **159**: 91–97

Lee, C. N., Wong, K. L., Liu, J. C., Chen, Y. J., Cheng, J. T., Chan, P. (2001) Inhibitory effect of stevioside on calcium influx to produce antihypertension. *Planta Med.* **67**: 796–799

Muller, S., Tirapelli, C. R., de Oliveira, A. M., Murillo, R., Castro, V., Merfort, I. (2003) Studies of *ent*-kaurane diterpenes from *Oyedaea verbesinoides* for their inhibitory activity on vascular smooth muscle contraction. *Phytochemistry* **63**: 391–396

Tirapelli, C. R., Ambrosio, S. R., Da Costa, F. B., de Oliveira, A. M. (2002) Inhibitory action of kaurenoic acid from *Viguiera robusta* (Asteraceae) on phenylephrine-induced rat carotid contraction. *Fitoterapia* **73**: 56–62

Tirapelli, C. R., Ambrosio, S. R., Da Costa, F. B., de Oliveira, A. M. (2003) Antispasmodic and relaxant actions of kaurenoic acid (KA) isolated from *Viguiera robusta* on rat carotid. *Braz. J. Pharm. Sci.* **39** (suppl. 2): 148

Tirapelli, C. R., Ambrosio, S. R., Da Costa, F. B., Coutinho, S. T., de Oliveira, D. C. R., de Oliveira, A. M. (2004) Analysis of the mechanisms underlying the vasorelaxant action of kaurenoic acid in the isolated rat aorta. *Eur. J. Pharmacol.* **492**: 233–241

Zamilpa, A., Tortoriello, J., Navarro, V., Delgado, G., Alvarez, L. (2002) Antispasmodic and antimicrobial diterpenic acids from *Viguiera hypargyrea* roots. *Planta Med.* **68**: 281–283