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Pharmacological comparison of the vasorelaxant action displayed by kaurenoic acid and pimaradienoic acid

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Abstract

The vascular effects of two natural occurring diterpenes from the kaurane and pimarane classes were compared. The diterpenes *ent*-kaur-16-en-19-oic acid (kaurenoic acid; KA) and *ent*-pimara-8(14),15-dien-19-oic acid (pimaradienoic acid; PA) were tested for their antispasmodic activity on isolated rat aorta. Vascular reactivity experiments, using standard muscle bath procedures, showed that KA and PA (both at 50 and 100 μM) inhibited phenylephrine and KCl-induced contraction in both endothelium-intact and endothelium-denuded rat aortic rings, with PA being more effective than KA. These compounds also reduced $CaCl_2$-induced contraction in Ca^{2+}-free solution containing KCl (30 mm). Again, PA produced a greater reduction in $CaCl_2$-induced contraction than KA. PA (1–300 μM) and KA (1–450 μM) concentration dependently relaxed endothelium-denuded aortic rings pre-contracted with KCl (maximum relaxation 102.31 ± 6.94% and 82.71 ± 1.40%, respectively). Similarly, the relaxation induced by KA on aortic rings pre-contracted with phenylephrine (73.06 ± 3.68%) was less pronounced than that found for PA (102.21 ± 3.64%). Incubation of endothelium-denuded rings for different periods showed that at 50 μM, KA and PA achieved maximum inhibitory activity on KCl-induced contraction after incubation for 60 (53.48 ± 5.83%) and 30 min (83.89 ± 2.12%), respectively. At 100 μM, KA and PA inhibited KCl-induced contraction, with a maximum after incubation for 30 min (73.58 ± 5.30% and 92.07 ± 1.20%, respectively). The maximum inhibition induced by PA at both concentrations tested was greater than that induced by KA. The results provide evidence that structural differences between diterpenes, independent of the C-19 carboxylic acid site, influence selectivity for voltage-operated Ca^{2+} channels and rate of equilibrium with the target site for their vasorelaxant action in rat aortic rings.

Introduction

Diterpenes from the kaurane and pimarane classes are reported to exert antispasmodic and relaxant actions on smooth muscle (Bejar et al 1984; Campos-Bedolla et al 1997; Ohashi et al 2000; Ambrosio et al 2002; Zamilpa et al 2000). We have previously demonstrated that the kaurane-type diterpene *ent*-kaur-16-en-19-oic acid (kaurenoic acid; KA) attenuated KCl and phenylephrine-induced contraction of rat carotid artery (Da Costa et al 2000; Tirapelli et al 2002). Moreover, KA relaxed segments of rat carotid pre-contracted with either phenylephrine or KCl (Tirapelli et al 2003). Using functional assays, we showed that KA blocked extracellular Ca^{2+} influx by interacting with both voltage- and receptor-operated channels. Its action also involves the stimulation of neuronal nitric oxide (NO) synthase and activation of the NO-cGMP pathway, which in turn could be responsible for the opening of K^+ channels (Tirapelli et al 2004a).

Ohashi et al (2000) reported that two migrated pimarane-type diterpenes and four isopimarane-type diterpenes exhibited suppressive effects on the contraction induced by KCl on rat thoracic aorta. Results from our laboratory showed that *ent*-pimara-8(14),15-dien-19-oic acid (pimaradienoic acid; PA) inhibited the contraction induced by phenylephrine or KCl on rat carotid artery (Ambrosio et al 2002). More recently, we described that the effects elicited by PA on vascular smooth muscle are endothelium-independent and involve extracellular Ca^{2+} influx blockade (Tirapelli et al 2004b). Moreover, the effects of PA are in part dependent on the release of NO and metabolites derived from the arachidonic acid pathway (Tirapelli et al 2004b).

It was previously assumed that the carboxylic group at C-19 played an important role in the biological activity of the kauranes, being responsible for the antispasmodic and relaxant action displayed by these compounds (Bejar et al 1984; Enriquez et al 1984; Campos-Bedolla et al 1997). However, recent data from our laboratory demonstrated that two kauranes that lack the carboxylic group also exerted antispasmodic activity on rat aorta (Muller et al 2003). Furthermore, we verified that the carboxylic group is not a prerequisite for the activity of KA since the methyl ester derivative obtained from the acid, in which a chemical blockade of the carboxylic group at C-19 was performed, also exhibits antispasmodic and relaxant actions (Ambrosio et al 2004).

It has been demonstrated that the inhibitory effect displayed by the kaurane *ent*-beyer-15-en-19-oic acid on electrically induced contractions of guinea-pig ileum was more pronounced than that found for KA (Zamilpa et al 2000). The structure of this compound is slightly different from 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. The other three rings (A, B and C) of both structures are identical. Ohashi et al (2000) described that the pimarane-type diterpenes orthosiphol A and B were more potent than neoorthosiphol A and B at inducing relaxation of aortic rings pre-contracted with KCl. The latter compounds are migrated pimare-type diterpenes, further indicating that differences in the chemical structure of these compounds alter their biological activity. Such findings have added new insights to studies of the biological activity of the diterpenes, indicating that portions of the chemical structure, other than the carboxylic group at C-19, are related to the antispasmodic action of diterpenes.

The chemical structures of KA and PA have many similarities. Both compounds have three rings (A, B and C), a carboxylic group at C-19 and two methyl groups at C-18 and C-20. However, KA differs from PA by the presence of a ring D, the absence of a methyl group attached at C-13 and the absence of a double bond between C-8 and C-14. In addition, a double bond between C-16 and C-17 is seen in KA. PA possesses a double bond between C-15 and C-16 that is not present in KA.

Based on the knowledge that KA and PA exert antispasmodic and relaxant activity on vascular smooth muscle and that both compounds show slight differences in their chemical structure, we decided to compare the vascular effects of these two naturally occurring diterpenes. A pharmacological comparison between the kaurane-type diterpene KA and the pimarane-type diterpene PA was therefore performed.

Materials and Methods

Drugs

Phenylephrine hydrochloride, acetylcholine hydrochloride, nifedipine and sodium nitroprusside were from Sigma (St Louis, MO, USA). Potassium chloride and calcium chloride were from Synth (São Paulo, Brazil). Nifedipine, KA and PA 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.

Isolation of KA

Individuals of *Viguiera robusta* Gardner were collected and authenticated by J. N. Nakajima (Universidade Federal de Uberlândia, Brazil) and Professor E. E. Schilling (Department of Botany, University of Tennessee, USA). The procedures for isolation of KA were carried out as previously described (Tirapelli et al 2002, 2004a). The purity (95–98%) of KA (colourless prisms, mp 129–130°C, $[a]_D^{25} = -110°$, $c = 1.5$ $CHCL_3$) was determined by thin-layer chromatography using different solvent systems, as well as gas chromatography and ^{13}C NMR spectral data.

Isolation of PA

Tuberous roots of *Viguiera arenaria* were collected and identified by Professor E. E. Schilling (Department of Botany, University of Tennessee, USA). The procedures for isolation of PA were carried out as previously described (Ambrosio et al 2002; Tirapelli et al 2004b). Isolation and purification steps were carried out by flash chromatography (hexane/EtOAc mixtures), preparative thin-layer chromatography (Si gel, hexane/EtOAc or hexane/$CHCl_3$) and recrystallization from MeOH. The structure of the diterpene was established by comparison of its ^1H and ^{13}C NMR spectral data with data reported in the literature (Shibata et al 1967; Matsuo et al 1976).

Vessel ring preparation

Male Wistar rats, 200–250 g (50–60 days old), were anaesthetized and killed by aortic exsanguination in accordance with the Ethical Animal Committee of the Campus of Ribeirão Preto, University of São Paulo, Brazil. The thoracic aorta was quickly removed, cut into rings (5–6 mm in length) and placed in a 5-mL organ chamber as previously described (Tirapelli et al 2004a). In some rings, the endothelium was removed mechanically by gently rolling the lumen of the vessel on a thin wire. Endothelial integrity was assessed in all vessels by the degree of relaxation caused by acetylcholine (1 μM) in the presence of contractile tone induced by phenylephrine (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 PA on contractions induced by phenylephrine, KCl and $CaCl_2$

Cumulative concentration–response curves for phenylephrine (10^{-10} to 10^{-5} M) or KCl (10–120 mM) were determined

in endothelium-intact and endothelium-denuded aortic rings. The curves for phenylephrine and KCl were determined in the absence (control) or after a 60-min incubation period with KA or PA (50 or 100 μM). To assess the effects of KA and PA on $CaCl_2$-induced contractions, endothelium-denuded rings were first contracted with phenylephrine (0.1 μM) to deplete the intracellular Ca^{2+} stores in Ca^{2+}-free solution (approx. 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 (control) or after a 60-min incubation period with KA or PA (50 or 100 μM).

Effect of KA and PA on aortic rings pre-contracted with phenylephrine or KCl

Steady tension was evoked by 0.03 μM phenylephrine or 30 mM KCl (to induce contractions of similar magnitude) and then KA (1–450 μM) or PA (1–300 μM) was added cumulatively. The relaxant effect of KA and PA was evaluated in endothelium-denuded rings. For comparison, the effect of sodium nitroprusside (10^{-10} to 10^{-8} M), a donor of NO, was also evaluated against the contractions induced by phenylephrine in endothelium-denuded rings. Relaxation was expressed as the percentage change from the KCl- or phenylephrine-contraction levels.

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

Endothelium-denuded rings were contracted with 30 mM KCl (control) and then washed out and pre-incubated with KA (50 or 100 μM) or PA (50 or 100 μM) for 15, 30, 60 or 90 min. Then, a further stimulation was performed with 30 mM KCl. The effect of the calcium channel blocker nifedipine (1 μM, 30 min) was also analysed for comparison. The stimulation with KCl was determined on the same ring, so that each ring served as its own control. Vessel rings from the same animal that were not exposed to the diterpenes or nifedipine served as time controls.

Statistical analysis

Two pharmacological parameters were analysed: the E_{max} (maximum effect generated by the agonist) and pD_2 (−log ED50). Results are expressed as means ± s.e.m. Statistical analysis of the E_{max} and pD_2 values was performed using one-way analysis of variance followed by Bonferroni comparison test (significance level = 0.05).

Results

Effect of KA and PA on contractions induced by phenylephrine, KCl and $CaCl_2$

The chemical structures of KA and PA are presented in Figure 1. The E_{max} values for phenylephrine and

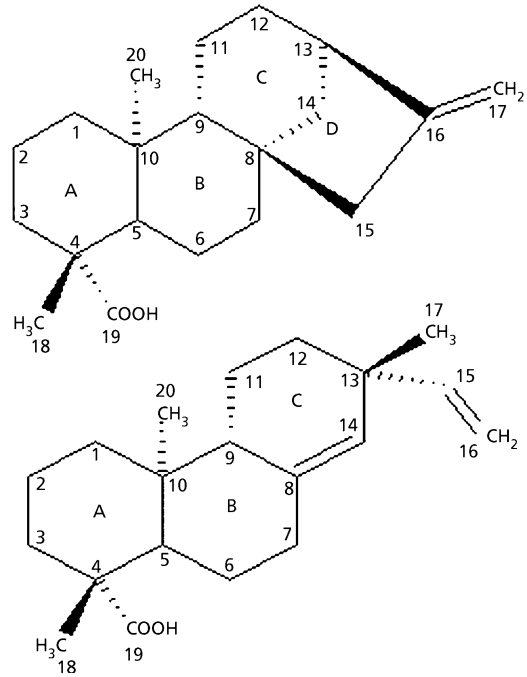

Figure 1 Chemical structures of kaurenoic acid (top) and pimaradienoic acid (bottom).

KCl in endothelium-intact or -denuded aortic rings were depressed in the presence of KA and PA at 50 and 100 μM (Figure 2; Table 1). At 100 μM, but not 50 μM, KA reduced the pD_2 values for phenylephrine in both endothelium-intact and -denuded rings. However, PA at 50 μM reduced the pD_2 values for phenylephrine in endothelium-denuded but not endothelium-intact rings. In the presence of 100 μM PA there was a reduction in the pD_2 values for phenylephrine in both endothelium-intact and -denuded rings. Both, KA and PA reduced the pD_2 values for KCl in endothelium-intact and -denuded rings (Figure 2; Table 1). As shown in Figure 3, pre-treatment with KA and PA attenuated $CaCl_2$-induced contraction of endothelium-denuded rat aorta. Both diterpenes at 50 or 100 μM significantly reduced the E_{max} values for $CaCl_2$ and produced a rightward displacement of the concentration–response curve for $CaCl_2$ (Table 2).

Effect of KA, PA and sodium nitroprusside on aortic rings pre-contracted with phenylephrine or KCl

The E_{max} values of the relaxant effect of KA for endothelium-denuded rings pre-contracted with either phenylephrine or KCl were significantly different from those obtained for PA. In aortic rings pre-contracted with KCl, but not in those pre-contracted with phenylephrine, there was a difference between the pD_2 values for KA and PA (Figure 4; Table 3). The relaxation induced by sodium nitroprusside in endothelium-denuded rings pre-contracted with phenylephrine was similar to that found for PA but significantly different compared with that found for KA (Table 3).

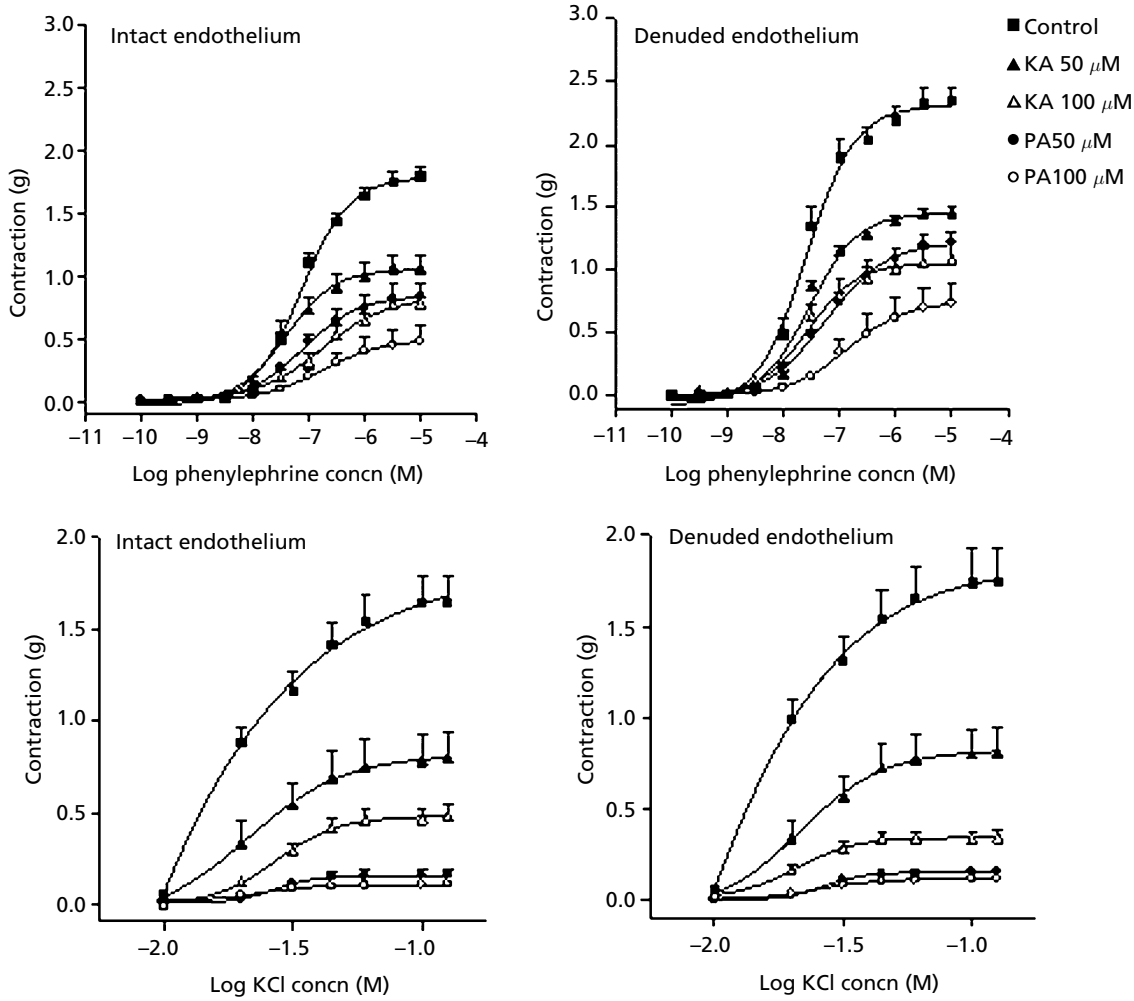

Figure 2 Effect of kaurenoic acid (KA) and pimaradienoic acid (PA) on phenylephrine- and KCl-induced contractile responses in rat aortic rings. Concentration–response curves for phenylephrine or KCl were determined in endothelium-intact or endothelium-denuded aortic rings. The curves were determined in the absence (control) of KA and PA or after a 60-min incubation with KA or PA (50 or 100 μM).

Table 1 Effect of kaurenoic acid (KA) and pimaradienoic acid (PA) on E_{max} (g) and pD_2 values for phenylephrine or KCl in endothelium-intact (Endo+) or -denuded (Endo−) aortic rings

	Diterpene	**Phenylephrine**		**KCl**	
		Endo+	Endo−	Endo+	Endo−
E_{max} (g)	Control (0 μM)	1.79 ± 0.08	2.35 ± 0.11	1.64 ± 0.14	1.74 ± 0.18
	KA 50 μM	1.06 ± 0.11^{a}	1.46 ± 0.06^{a}	0.79 ± 0.14^{a}	0.81 ± 0.13^{a}
	KA 100 μM	$0.78 \pm 0.05^{a,b}$	$1.10 \pm 0.14^{a,b}$	$0.48 \pm 0.06^{a,b}$	$0.34 \pm 0.05^{a,b}$
	PA 50 μM	$0.84 \pm 0.11^{a,b}$	$1.22 \pm 0.09^{a,b}$	$0.15 \pm 0.03^{a,b,c}$	$0.16 \pm 0.03^{a,b,c}$
	PA 100 μM	$0.48 \pm 0.13^{a,b,c,d}$	$0.73 \pm 0.16^{a,b,c,d}$	$0.11 \pm 0.02^{a,b,c}$	$0.12 \pm 0.03^{a,b,c}$
pD_2	Control (0 μM)	7.13 ± 0.06	7.56 ± 0.08	1.75 ± 0.10	1.73 ± 0.09
	KA 50 μM	7.27 ± 0.14	7.58 ± 0.06	1.52 ± 0.09^{a}	1.53 ± 0.11^{a}
	KA 100 μM	$6.89 \pm 0.15^{a,b}$	$7.28 \pm 0.10^{a,b}$	1.41 ± 0.09^{a}	1.39 ± 0.09^{a}
	PA 50 μM	7.11 ± 0.06^{c}	$7.24 \pm 0.04^{a,b}$	1.58 ± 0.05^{a}	1.59 ± 0.04^{a}
	PA 100 μM	$6.85 \pm 0.11^{a,b,d}$	$7.01 \pm 0.18a^{a,b,c}$	1.61 ± 0.04^{a}	1.62 ± 0.04^{a}

Values are means ± s.e.m., n = 5–9 experiments. [a]Significantly different compared with the respective control; [b]significantly different compared with KA 50 μM; [c]significantly different compared with KA 100 μM; [d]significantly different compared with PA 50 μM; analysis of variance followed by Bonferroni's multiple comparison test, $P < 0.05$.

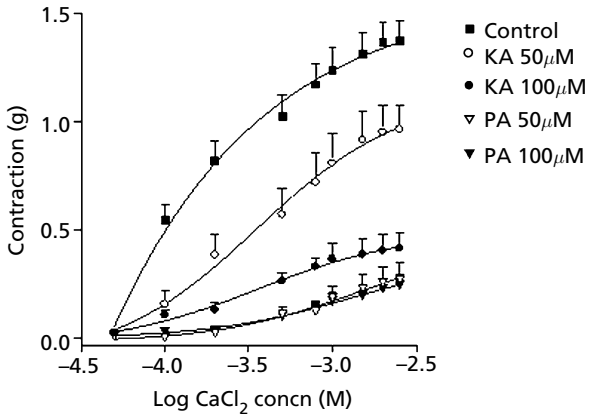

Figure 3 Effect of kaurenoic acid (KA) and pimaradienoic acid (PA) on the $CaCl_2$-induced contractile response in endothelium-denuded aortic rings. Concentration–response curves for $CaCl_2$ were determined in Ca^{2+}-free solution containing KCl (30 mM). The curves were determined in the absence (control)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

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

No differences in the E_{max} values induced by 30 mM KCl in time control experiments were detected (data not shown). KA and PA inhibited the contraction induced by KCl (30 mM) in a time- and concentration-dependent manner. At 50 μM, KA and PA achieved their maximum inhibitory activity after a pre-incubation period of 60 and 30 min, respectively; the effect of PA was more pronounced than that found for KA. At 100 μM, KA and PA inhibited KCl-induced contraction, with a maximum after incubation for 30 min. Nifedipine was more potent than the diterpenes at inhibiting KCl-induced contractions (Table 4).

Discussion

The present findings corroborate our previous results, in particular that KA and PA display antispasmodic and relaxant effects on rat isolated carotid rings (Ambrosio et al 2002; Tirapelli et al 2002) and aorta (Tirapelli et al 2004a, b). Our data show that KA and PA concentration-dependently reduced the contractions induced by KCl and the α_1-selective agonist phenylephrine in endothelium-intact and -denuded aortic rings. However, at both concentrations tested, the inhibitory effect of PA on phenylephrine- and KCl-induced contractions was more pronounced than that found for KA in both endothelium-intact and -denuded rings. At 50 μM, PA, but not KA, reduced the potency of phenylephrine on endothelium-denuded rings. Taken together, these results support the idea that PA is more effective than KA at inhibiting phenylephrine- and KCl-induced contractions.

Our findings show that in the presence of KA and PA there was a rightward shift in the concentration–response curves for $CaCl_2$, with a decrease in the E_{max} values. In the present study, the concentration–response curves for $CaCl_2$ were obtained in Ca^{2+}-free medium containing KCl. It is well established that contractions of rat aortic rings induced by KCl rely almost exclusively on Ca^{2+} influx through activation of voltage-sensitive channels (Hudgins & Weiss 1968). The results indicate that both compounds can block Ca^{2+} influx through Ca^{2+} channels, as previously shown (Tirapelli et al 2004a, b). However, we found that at 50 or 100 μM, KA produced a less accentuated reduction in the E_{max} values for $CaCl_2$ than that induced by PA at the same concentrations. Furthermore, PA at 50 μM produced a more pronounced rightward displacement of the concentration–response curves for $CaCl_2$ than KA. These results indicate that PA is more potent than KA at inhibiting extracellular Ca^{2+} influx.

In addition to their antispasmodic activity, the diterpenes relaxed rings pre-contracted with phenylephrine or KCl. The pD_2 values obtained for sodium nitroprusside, a NO donor, on endothelium-denuded rings pre-contracted with phenylephrine were greater when compared with those found for the diterpenes, further indicating that this compound is more potent than KA and PA. On the other hand, the E_{max} values for sodium nitroprusside were greater than that found for KA but not for PA. Thus, it can be concluded that sodium nitroprusside is more potent than these compounds but is as effective as PA at

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

	Control	KA		PA	
		50 μM	100 μM	50 μM	100 μM
E_{max}	1.37 ± 0.10	0.96 ± 0.11^{a}	$0.41 \pm 0.08^{a,b}$	$0.27 \pm 0.07^{a,b}$	$0.25 \pm 0.06^{a,b,c}$
pD_2	3.89 ± 0.14	3.51 ± 0.11^{a}	$3.20 \pm 0.10^{a,b}$	$3.00 \pm 0.07^{a,b}$	$3.08 \pm 0.16^{a,b}$

Values are means $\pm$ s.e.m., n = 6–8 experiments. [a]Significantly different compared with the control; [b]significantly different compared with KA 50 μM; [c]significantly different compared with KA 100 μM; analysis of variance followed by Bonferroni's multiple comparison test, $P < 0.05$.

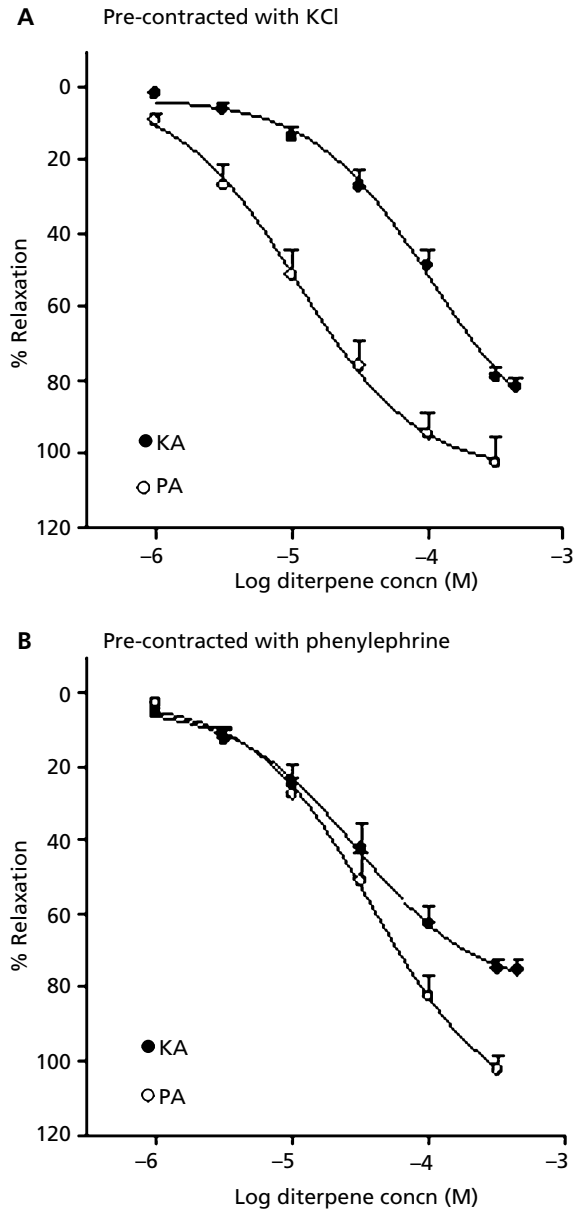

Figure 4 Relaxation responses induced by kaurenoic acid (KA) and pimaradienoic acid (PA) on rat aortic rings pre-contracted with KCl (A) or phenylephrine (B). The relaxation induced by the diterpenes was determined in endothelium-denuded rat aortic rings submaximally pre-contracted with either KCl (30 mM) or phenylephrine (0.03 μM). Steady tension was evoked by phenylephrine or KCl and then KA (1–450 μM) or PA (1–300 μM) were added cumulatively.

inducing relaxation. It is important to note that KA and PA relaxed aortic rings pre-contracted with phenylephrine, with similar pD_2 values (i.e. the same potency). Conversely, PA was more potent than KA at producing relaxation of aortic rings pre-contracted with KCl. The contractions induced by phenylephrine are mediated by an increase in Ca^{2+} influx through both receptor-operated channels (Hirata et al 1998) and voltage-sensitive channels (Wesselman et al 1996; Lee et al 2001), while KCl-induced contractions are mediated mainly by Ca^{2+} influx through activation of voltage-sensitive channels (Hudgins & Weiss 1968). This corroborates our initial observation that PA is more effective at inhibiting KCl-induced contraction than phenylephrine-induced contraction and suggests that PA is more selective at inhibiting the Ca^{2+} influx through activation of voltage-sensitive channels, as previously described (Tirapelli et al 2004b). In addition, our results show that the relaxation induced by PA is greater than that induced by KA in aortic rings pre-contracted with either phenylephrine or KCl. This observation allows us to propose that the structural differences between these two compounds are responsible for the enhanced activity of PA and its selectivity at inhibiting Ca^{2+} influx through voltage-sensitive channels. However, it is important to note that the inhibition of Ca^{2+} influx is not the only mechanism involved in the vascular effects of these diterpenes. KA-induced relaxation also involves the activation of the NO-cGMP pathway and the opening of K^+ channels (Tirapelli et al 2004a), while PA-induced relaxation does not involve the opening of K^+ channels but is related to the production of a vasodilatory arachidonic acid metabolite (Tirapelli et al 2004b). Thus, the differences in the mechanism of action displayed by these compounds could account for the difference in their vascular relaxation.

We previously provided evidence that the equilibrium period is a critical step for the inhibitory effect displayed by kaurane-type diterpenes (Muller et al 2003; Ambrosio et al 2004). In the present work, the maximum inhibition induced by KA and PA at 50 μM on KCl-induced contraction was observed after 60 and 30 min of incubation, respectively. Moreover, at this concentration, PA induced a more pronounced inhibition than that found for KA. At the higher concentration (100 μM), both compounds achieved maximum inhibition after 30 min of incubation. Again, PA displayed more pronounced inhibitory activity than KA. These results show that the equilibrium period for PA to achieve its maximum response is shorter than that for KA. It is also important to note that the inhibitory effect displayed by PA is more pronounced than that found for KA. Furthermore, after incubation for 15 min, the inhibitory effect displayed by PA at both concentrations was much greater than that found for KA. Such findings indicate that structural differences between these compounds potentiate and accelerate the inhibitory activity displayed by PA.

Zamilpa et al (2000) demonstrated that the inhibitory effect displayed by *ent*-beyer-15-en-19-oic acid on electrically induced contractions of guinea-pig ileum was more pronounced than that found for KA. The structure of this compound is slightly different from that of KA because of some differences in ring D. The other three rings (A, B and C) of both structures are identical. Moreover, Ohashi et al (2000) described that neoorthosiphol A and B, two migrated pimares, were less potent than the pimaranes orthosiphol A and B at inducing relaxation of aortic rings pre-contracted with KCl, further indicating that alterations at ring C alter the biological activity of these compounds. Our data is in line with these previous observations, since PA, which possesses some structural differences at ring C, such as the presence of a double bond

Table 3 Effect (% relaxation) of kaurenoic acid (KA), pimaradienoic acid (PA) and sodium nitroprusside on endothelium-denuded aortic rings pre-contracted with phenylephrine or KCl

	KA		PA		Sodium nitroprusside	
	E_{max}	pD_2	E_{max}	pD_2	E_{max}	pD_2
KCl	82.71 ± 1.40	3.98 ± 0.12	$102.31 \pm 6.94^{a,b}$	$4.98 \pm 0.11^{a,b}$	–	–
Phenylephrine	73.06 ± 3.68^{a}	4.52 ± 0.12^{a}	$102.21 \pm 3.64^{a,b}$	$4.39 \pm 0.13^{a,c}$	$106.51 \pm 3.27^{a,b}$	$8.51 \pm 0.15^{a,b,c,d}$

Values are means ± s.e.m., n = 8–12 experiments. [a]Significantly different compared with KA (pre-contracted with KCl); [b]significantly different compared with KA (pre-contracted with phenylephrine); [c]significantly different compared with PA (pre-contracted with KCl); [d]significantly different compared with PA (pre-contracted with phenylephrine); analysis of variance followed by Bonferroni's multiple comparison test, $P < 0.05$.

Table 4 Percentage inhibition induced by kaurenoic acid (KA), pimaradienoic acid (PA) and nifedipine on KCl-induced contraction of endothelium-denuded rat aorta after different incubation times

Incubation time (min)	KA		PA		Nifedipine
	50 μM	100 μM	50 μM	100 μM	1 μM
15	8.02 ± 2.20^{a}	42.70 ± 3.11^{a}	$69.62 \pm 3.35^{a,e}$	$76.20 \pm 1.77^{a,f}$	–
30	$17.32 \pm 5.97^{a,b}$	$73.58 \pm 5.30^{a,d}$	$83.89 \pm 2.12^{a,b,e}$	$92.07 \pm 1.20^{a,d,f}$	81.23 ± 1.92^{a}
60	$53.48 \pm 5.83^{a,b,c}$	$75.64 \pm 4.00^{a,d}$	$88.72 \pm 3.21^{a,b,e}$	$92.85 \pm 1.06^{a,d,f}$	–
90	$56.50 \pm 4.63^{a,b,c}$	$77.65 \pm 4.20^{a,d}$	$85.77 \pm 1.98^{a,b,e}$	–	–

Values are means ± s.e.m., n = 6–8 experiments. The rings were initially stimulated with 30 mM KCl (control, 100% contraction) and a second stimulation was performed after incubation with two concentrations of KA, PA or nifedipine. [a]Significantly different compared with the respective control (0% inhibition); [b]significantly different compared with pre-incubation for 15 min at 50 μM; [c]significantly different compared with pre-incubation for 30 min at 50 μM; [d]significantly different compared with pre-incubation for 15 min at 100 μM, [e]significantly different compared with the respective period of incubation at 50 μM; [f]significantly different compared with the respective period of incubation at 100 μM; analysis of variance followed by Bonferroni's multiple comparison test, $P < 0.05$.

between C-15 and C-16, had more pronounced antispasmodic and relaxant activity than KA.

It has been reported that the carboxylic group at C-19 is the most important group in the structure of the diterpenes, being responsible for the antispasmodic activity displayed by these compounds (Bejar et al 1984; Enriquez et al 1984; Campos-Bedolla et al 1997). However, kauranes, which lack the carboxylic group (Muller et al 2003), or have had this group blocked (Ambrosio et al 2004), also reduced the contraction of rat aorta, supporting the view that the carboxylic group is not a prerequisite for the antispasmodic activity exerted by these diterpenes. The present data corroborate these observations since the carboxylic group at C-19 is present in the structure of KA and PA. Based on the present findings, we can also conclude that the carboxylic group at C-19 is not the only component of the structure of the diterpenes responsible for their antispasmodic and relaxant effect.

Considering their vascular effects, it is possible to suggest that KA and PA could potentially exert antihypertensive action in-vivo. It is therefore important to understand the structure–activity relationships of these compounds since structural alterations can improve their biological activity. The present work brings new perspectives to studies of the biological activity of diterpenes, indicating that differences in the chemical structure, other than the carboxylic group at C-19, can improve the antispasmodic action displayed by these compounds.

Conclusion

From our data we conclude that structural differences between diterpenes, independent of the C-19 carboxylic acid site, influence selectivity for voltage-operated Ca^{2+} channels and rate of equilibrium with the target site for their vasorelaxant action in rat aortic rings. In addition, the chemical structure of ring C plays an important role in the antispasmodic activity displayed by diterpenes.

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