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Analysis of the mechanisms underlying the vasorelaxant action of kaurenoic acid in the isolated rat aorta

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Abstract

The present work describes the mechanisms involved in the vasorelaxant effect of the diterpene *ent*-kaur-16-en-19-oic acid (kaurenoic acid). Kaurenoic acid (10, 50 and 100 μM) concentration-dependently inhibited phenylephrine and KCl-induced contraction in either endothelium-intact or -denuded rat aortic rings. Kaurenoic acid also reduced $CaCl_2$-induced contraction in Ca^{2+}-free solution containing KCl (30 mM). The diterpene did not interfere with Ca^{2+} release from intracellular stores mediated by either phenylephrine (1 μM) or caffeine (30 mM). Kaurenoic acid (1–450 μM) concentration dependently relaxed phenylephrine-pre-contracted rings with intact (72.27 ± 3.79%) or denuded endothelium (73.28 ± 5.91%). The diterpene also relaxed KCl-pre-contracted rings with intact (80.44 ± 3.68%) or denuded endothelium (78.12 ± 1.26%). Pre-incubation of denuded aortic rings with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μM) and 7-nitroindazole (100 μM) reduced kaurenoic acid-induced relaxation (percentage of relaxation: 49.12 ± 3.26%, 53.10 ± 6.72% and 51.74 ± 4.76%, respectively). Indomethacin (10 μM) did not affect kaurenoic acid-induced relaxation. In endothelium-intact rings, 7-nitroindazole and N^{ϖ}-nitro-L-arginine (L-NNA, 100 μM) displaced the curves for the diterpene to the right. Tetraethylammonium (5 mM), 4-amynopiridine (1 mM) and charybdotoxin (0.1 μM) caused a rightward displacement of the concentration–response curve for kaurenoic acid. Conversely, neither apamin (1 μM) nor glibenclamide (3 μM) affected kaurenoic acid-induced relaxation. Collectively, our results provide functional evidence that the effects elicited by kaurenoic acid involve extracellular Ca^{2+} influx blocked. Its effects are also partly mediated by the activation of NO–cGMP pathway and the opening of K^+ channels sensitive to charybdotoxin and 4-amynopiridine. Additionally, the activation of the endothelial and neuronal NO synthase isoforms are required for the relaxant effect induced by kaurenoic acid.

Keywords: Kaurenoic acid; Relaxation; Aorta, rat; K^+ channel; Ca^{2+} influx

1. Introduction

Biological assays have shown that kaurane-type diterpenes exert antispasmodic and relaxant actions on smooth muscle. Bejar et al. (1984) described that kauradienoic acid abolished the spontaneous contractile activity of rat uterine tissue and inhibited the contraction induced by prostaglandin $F_{2\alpha}$, acetylcholine and oxytocin. These authors have also described that the kauradienoic acid relaxed uterine strips pre-contracted with Ca^{2+}, suggesting that the diterpene could reduce extracellular Ca^{2+} influx. kauradienoic acid was also described to display inhibitory action upon spontaneous contractility of the rat, guinea pig and human uteri (Enriquez et al., 1984).

Kaurenoic acid (*ent*-kaur-16-en-19-oic acid) and its closely related derivatives, kauradienoic acid, grandifloric acid and 16α-Hydroxy-ent-kauran-19-oic acid, displayed

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inhibitory effect on uterine contraction induced by acetylcholine, oxytocin and serotonin (Campos-Bedolla et al., 1997). Recently, kaurenoic acid was described to inhibit the electrically induced contractions of guinea pig ileum (Zamilpa et al., 2002).

We have previously demonstrated that kaurenoic acid exhibits antispasmodic action on vascular smooth muscle. The diterpene inhibited the contraction induced by a single concentration of KCl and reduced the contraction induced by phenylephrine in the isolated rat carotid artery with intact endothelium (Da Costa et al., 2000; Tirapelli et al., 2002). More recently, we have provided evidence that kaurenoic acid relaxes segments of endothelium-intact rat carotid precontracted with either phenylephrine or KCl (Tirapelli et al., 2003).

Although it has been described that kaurane-type diterpenes exert antispasmodic and relaxant actions on smooth muscle, the mechanisms underlying these effects are poorly understood. In the present study, we aimed to investigate the mechanism(s) involved in the vasorelaxant action of kaurenoic acid in rat isolated aortic rings.

2. Material and methods

2.1. Procedure for isolation of kaurenoic acid

Individuals of *Viguiera robusta* Gardner were collected and authenticated by J.N. Nakajima (Universidade Federal de Uberlândia, Brazil) and E.E. Schilling (University of Tennessee, USA). Vouchers specimens (reg. number FBC 60) are deposited in the herbarium of the Departamento de Biologia, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil. Air-dried (40 °C) powdered roots (3 g) were macerated with CH_2Cl_2 (150 ml) in sonicator at room temperature for 15 min. After solvent evaporation under vacuum, the crude extract (550 mg) was analysed by thin layer chromatography and infra-red spectroscopy. According to previous work (Da Costa et al., 1996), this preliminary analysis indicated the presence of diterpene acids. A gas chromatography analysis (Hewlett Packard 5890-Series II equipment; 30 m × 0.25 mm, 0.25 μm HP1 capillary column; FID detector temperature 300 °C, injector temperature 240 °C; oven temp. 105–200; 200–240; 240–280 °C) has been performed with a previously cleaned-up aliquot of the crude extract. Natural diterpenes were used as standard compounds and kaurenoic acid (*ent*-kaur-16-en-19-oic acid) was detected in the extract. 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 thin layer chromatography analysis. Fraction 3 (100 mg) furnished 20 mg of pure kaurenoic acid which was isolated by prep. thin layer chromatography (silica gel, Merck; *n*-hexane–EtOAc 4:1) and identified by means of spectrometric analysis (infra-red and nuclear magnetic resonance), comparison with authentic sample and data from literature (Da Costa et al., 1996). The purity (95–98%) of kaurenoic acid (colorless prism, m.p. 129–130 °C, $[\alpha]_D^{25} = -110$ °, $c = 1.5$ $CHCL_3$) was determined by thin layer chromatography analysis using different solvent systems as well as gas chromatography and ^{13}C nuclear magnetic resonance spectral data.

2.2. Vessel ring preparation

Male Wistar rats weighting between 200 and 250 g (50–60 days old) were anaesthetized with ether 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 until an optimal basal tension of 1.0 g, which was determined by length–tension relationship experiments and then were 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 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.

2.3. Effect of kaurenoic acid on contractions induced by phenylephrine, KCl and $CaCl_2$

After equilibration, cumulative concentration–response curves for phenylephrine (10^{-10}–10^{-5} M) and KCl (10–90 mM) were determined. The curves were obtained in intact and denuded rings by a stepwise increase in the concentration of phenylephrine or KCl. Additions were made as soon as a steady response was obtained from the preceding concentration. The curves for phenylephrine or KCl were determined in the absence (control group) or after a 60-min incubation period with kaurenoic acid (10, 50 or 100 μM).

To assess the effects of kaurenoic acid 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 (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 (control group) or after a 60-min incubation period with kaurenoic acid (10, 50 or 100 μM).

2.4. Influence of kaurenoic acid on Ca^{2+} release from intracellular stores sensitive to phenylephrine and caffeine

In order to investigate whether kaurenoic acid could interfere with Ca^{2+} release from intracellular stores, the normal Krebs solution was replaced for a Ca^{2+}-free solution containing EGTA (1 mM). The rings were exposed to this solution for 1 min (David et al., 2002) and then were stimulated with phenylephrine 1 μM or a solution of caffeine 30 mM. The contractions for both agonists were obtained in the absence (control group) or after a 60-min incubation period with kaurenoic acid (10, 50 or 100 μM).

2.5. Effect of kaurenoic acid on aortic rings contracted with phenylephrine or KCl

In another set of experiments, steady tension was evoked by phenylephrine (0.1 μM for endothelium-intact rings and 0.03 μM for endothelium-denuded rings to induced contraction of similar magnitude) and then kaurenoic acid was added cumulatively (1–450 μM). The effect of kaurenoic acid on attenuating KCl-induced sustained contraction (30 mM) in endothelium-intact or -denuded rings was also examined. 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 phenylephrine and KCl in endothelium-denuded rings.

To investigate the possible mechanism(s) responsible for kaurenoic acid-induced relaxation, the preparations without endothelium were contracted with phenylephrine (0.03 μM) 30 min after being incubated with one of the following drugs: N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 1 μM), indomethacin (10 μM), aminoguanidine (100 μM), 7-nitroindazole (100 μM), N^{ω}-nitro-L-arginine (L-NNA, 100 μM), tetraethylammonium, (5 mM), 4-amynopiridine (1 mM), apamin (1 μM), glibenclamide (3 μM) and charybdotoxin (0.01 μM).

To account possible overlapping mechanisms in the relaxant effect of kaurenoic acid, the NO synthase inhibitors were tested in the rings with endothelium. The preparations were contracted with phenylephrine 30 min after being incubated with aminoguanidine (100 μM), 7-nitroindazole (100 μM) or L-NNA (100 μM). Relaxation was expressed as percentage change from the phenylephrine-contracted levels.

2.6. Drugs

The following drugs were used: phenylephrine hydrochloride, acetylcholine hydrochloride, nifedipine, ODQ, glibenclamide, 4-amynopiridine, L-NNA, 7-nitroindazole, aminoguanidine (Sigma, St. Louis, MO, USA), potassium chloride and calcium chloride (Synth, São Paulo, Brazil), L-NAME, tetraethylammonium (Sigma/RBI, Natick, MA), indomethacin (Calbiochem), apamin, charybdotoxin (American Peptide). Nifedipine, glibenclamide and kaurenoic acid were prepared as stock solutions in ethanol. ODQ and 7-nitroindazole were prepared as stock solution 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 ethanol or 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.

2.7. Statistical analysis

To study the effect of kaurenoic acid on attenuating contraction or inducing relaxation, two pharmacological parameters were analysed: the E_{max} (maximal effect generated by the agonist) and pD_2 ($-\log EC_{50}$). Results were expressed as means ± standard error of the mean (S.E.M.). Statistical analysis of the E_{max} values was performed using one-way analysis of variance (ANOVA) or Student's *t*-test. The same analysis was applied to pD_2 values. The contractions (g) induced by phenylephrine or caffeine in Ca^{2+}-free solution obtained in the experiments, designed to investigate whether kaurenoic acid could interfere with Ca^{2+} release from intracellular stores, were performed using ANOVA. Post hoc comparisons were performed after ANOVA analysis using Bonferroni or Dunnet test as indicated in the text and tables. The significance level considered in all tests was 0.05.

3. Results

3.1. Effect of kaurenoic acid on contractions induced by phenylephrine, KCl and $CaCl_2$

The chemical structure of kaurenoic acid is represented in Fig. 1. The effects of kaurenoic acid on the cumulative concentration–response curves for phenylephrine and KCl on isolated rat aorta are shown in Fig. 2 and Table 1A and B. The E_{max} values for phenylephrine or KCl in endothelium-intact or -denuded rings were depressed in the presence of kaurenoic acid at 50 and 100 μM. Kaurenoic acid did not alter the pD_2 values for phenylephrine in either endothelium-intact or -denuded rings. On the other hand, there was a reduction in the pD_2 values for KCl in the presence of kaurenoic acid at 100 μM in both intact and denuded rigs. The pD_2 values for KCl in denuded rings reduced in the presence of kaurenoic acid at 50 μM.

As it can be seen in Fig. 3, pretreatment with kaurenoic acid attenuated $CaCl_2$-induced contraction of denuded rat aorta exposed to Ca^{2+}-free medium containing KCl. $CaCl_2$ induced a concentration-dependent contraction of rat aortic

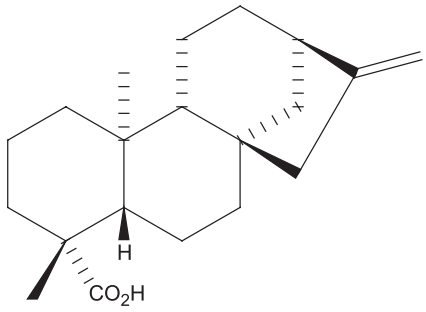

Fig. 1. Chemical structure of *ent*-kaur-16-en-19-oic acid (kaurenoic acid).

rings ($E_{max} = 1.47 \pm 0.11$ g; $pD_2 = 4.12 \pm 0.19$; $n = 7$). Pre-incubation of the rings with kaurenoic acid at 10, 50 or 100 μM significantly reduced the E_{max} values for $CaCl_2$ ($E_{max} = 0.92 \pm 0.10$ g, 0.89 ± 0.13 g, 0.52 ± 0.11 g, respectively; $n = 5$ for each group). Similarly, the pD_2 values for $CaCl_2$ were reduced in the presence of KA at 50 or 100 μM ($pD_2 = 3.03 \pm 0.14$ and 3.48 ± 0.12, respectively). On the other hand, kaurenoic acid at 10 μM did not alter the pD_2 values (4.25 ± 0.20) for $CaCl_2$ (ANOVA followed by Bonferroni's multiple comparison test).

3.2. Influence of kaurenoic acid on Ca^{2+} release from intracellular stores

The results presented in Table 2 show that the contractions induced by either phenylephrine or caffeine in Ca^{2+}-free solution containing EGTA were not affected by kaurenoic acid.

3.3. Effect of kaurenoic acid on aortic rings pre-contracted with phenylephrine or KCl

Kaurenoic acid at concentrations ranging from 1 to 450 μM significantly inhibited the sustained tonic contraction induced by phenylephrine and KCl in a concentration-dependent manner (Fig. 4). The E_{max} values (percentage of relaxation) of the relaxant effect of kaurenoic acid for the intact and denuded rings pre-contracted with phenylephrine were not significantly different ($72.27 \pm 3.79\%$ and $73.28 \pm 5.91\%$, respectively). Similarly, no differences were found in the pD_2 values for kaurenoic acid in intact or denuded rings (4.29 ± 0.18 and 4.56 ± 0.20; respectively). In the arteries pre-contracted with KCl, there was no difference between the E_{max} values for kaurenoic acid in

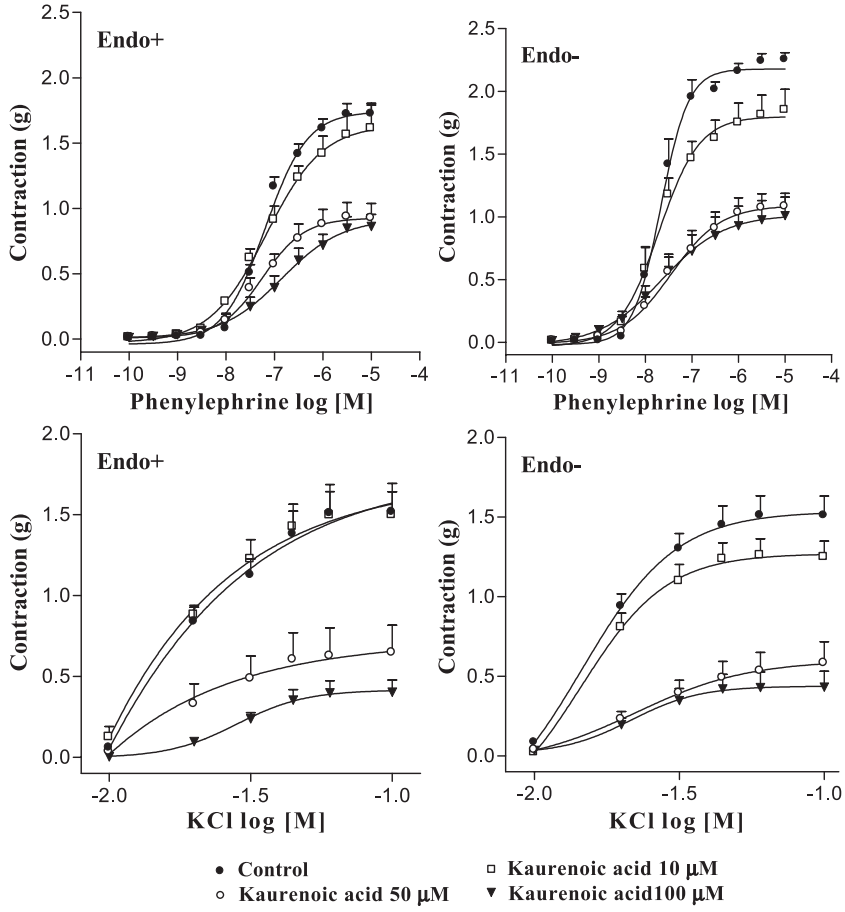

Fig. 2. Effect of kaurenoic acid on phenylephrine and KCl-induced contractile response in rat aortic rings. Concentration–response curves for both agonists were determined in endothelium-intact (Endo+) or endothelium-denuded (Endo −) rings. The curves were determined in the absence (control) or after a 60-min period of incubation with kaurenoic acid (10, 50 or 100 μM).

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

Kaurenoic acid (μM)	Phenylephrine		KCl	
	Endo+	Endo −	Endo+	Endo −
A				
0	1.74 ± 0.09	2.28 ± 0.06	1.87 ± 0.26	1.80 ± 0.14
10	1.62 ± 0.14	1.82 ± 0.15	1.77 ± 0.18	1.54 ± 0.14
50	0.94 ± 0.11[a,b]	1.08 ± 0.10[a,b]	0.94 ± 0.12[a,b]	0.76 ± 0.18[a,b]
100	0.87 ± 0.09[a,b]	0.96 ± 0.14[a,b]	0.62 ± 0.13[a,b]	0.55 ± 0.13[a,b]
B				
0	7.19 ± 0.06	7.65 ± 0.14	6.35 ± 0.24	7.13 ± 0.17
10	7.12 ± 0.17	7.72 ± 0.10	6.60 ± 0.22	7.10 ± 0.22
50	7.23 ± 0.16	7.37 ± 0.20	7.23 ± 0.16	5.05 ± 0.22[a,b]
100	6.96 ± 0.24	7.58 ± 0.12	4.73 ± 0.11[a,b,c]	5.56 ± 0.27[a,b]

Values are means ± S.E.M., $n = 6–8$ experiments.
[a] Compared to control group (ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$).
[b] Compared to kaurenoic acid 10 μM (ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$).
[c] Compared to kaurenoic acid 50 μM (ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$).

intact or denuded rings (80.44 ± 3.68% and 78.12 ± 1.26%, respectively). The E_{max} values found for kaurenoic acid in the rings pre-contracted with KCl were not different from those found in phenylephrine-pre-contracted rings. The pD_2 values for kaurenoic acid in intact and denuded rings pre-contracted with KCl were not significantly different (4.30 ± 0.24 and 3.96 ± 0.16, respectively), being not different from those found in phenylephrine-pre-contracted rings (ANOVA followed by Bonferroni's multiple comparison test).

It can be seen in Fig. 4 that the relaxation induced by nifedipine ($n = 7$, 88.84 ± 4.00%) in denuded rings pre-contracted with phenylephrine was significantly different from

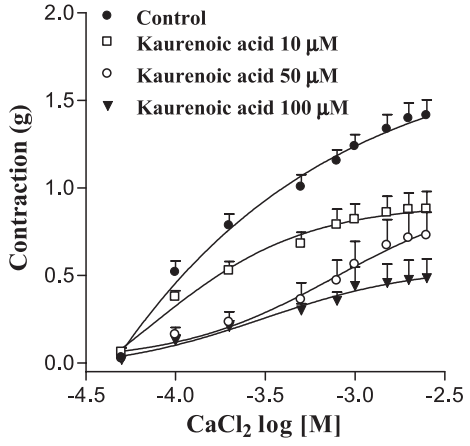

Fig. 3. Effect of kaurenoic acid on $CaCl_2$-induced contractile response in endothelium-denuded aortic rings. Concentration–response curves for $CaCl_2$ were determined in Ca^{2+}-free solution containing KCl (30 mM). The curves were determined in the absence (control), after a 60-min period of incubation with kaurenoic acid (10, 50 or 100 μM).

Table 2
Effect of kaurenoic acid on phenylephrine (1 μM) and caffeine (30 mM)-induced contraction (g) in Ca^{2+}-free medium

Contractile agent	Kaurenoic acid (μM)			
	0	10	50	100
Phenylephrine	0.55 ± 0.04	0.54 ± 0.07	0.51 ± 0.06	0.55 ± 0.12
Caffeine	0.30 ± 0.02	0.29 ± 0.05	0.26 ± 0.07	0.28 ± 0.03

Values are means ± S.E.M., $n = 6–8$ experiments (ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$).

those found for kaurenoic acid (73.28 ± 5.91%). The pD_2 values found for nifedipine (pD_2 = 6.31 ± 0.17) were higher than that found for kaurenoic acid (pD_2 = 4.56 ± 0.20). Nifedipine also exerted a more pronounced relaxation ($n = 7$, 98.90 ± 2.01%) in denuded KCl-pre-contracted aortic rings when compared with kaurenoic acid (78.12 ± 1.26%). Again, the pD_2 values found for nifedipine (pD_2 = 7.34 ± 0.17) were higher than that found for kaurenoic acid (pD_2 = 3.96 ± 0.16) (Student's *t*-test).

The experiments designed to investigate the mechanisms responsible for kaurenoic acid-induced relaxation were conducted in endothelium-denuded rings pre-contracted with phenylephrine. The NO synthase inhibitor L-NAME, as well as ODQ, reduced kaurenoic acid-induced relaxation and produced a rightward displacement of the concentration–response curve for the diterpene. On the other hand, indomethacin had not significant effect on kaurenoic acid-induced relaxation. The combination of L-NAME and indomethacin showed no further suppression than that observed with L-NAME alone. Aminoguanidine as well as L-NNA had no effect on kaurenoic acid-induced relaxation. Conversely, 7-nitroindazole reduced the relaxation induced by kaurenoic acid and produced a rightward displacement of the concentration–response curve for the diterpene (Fig. 5; Table 3).

In endothelium-intact rings, 7-nitroindazole significantly reduced the contraction induced by kaurenoic acid. In addition, 7-nitroindazole and L-NNA displaced the concentration–response curves to the right. However, aminoguanidine did not alter kaurenoic acid-induced relaxation (Fig. 6; Table 4).

Pre-incubation of the rings with tetraethylammonium produced a rightward displacement of the concentration–response curves for kaurenoic acid. Similarly, 4-amynopiridine as well as charybdotoxin also caused a rightward displacement of the concentration–response curve for the diterpene. On the other hand, apamin and glibenclamide did not alter the relaxant effect of kaurenoic acid (Fig. 7; Table 5).

4. Discussion

The present findings corroborate those of our previous study conducted in rat isolated carotid, namely that kaurenoic acid displays antispasmodic (Da Costa et al., 2000;

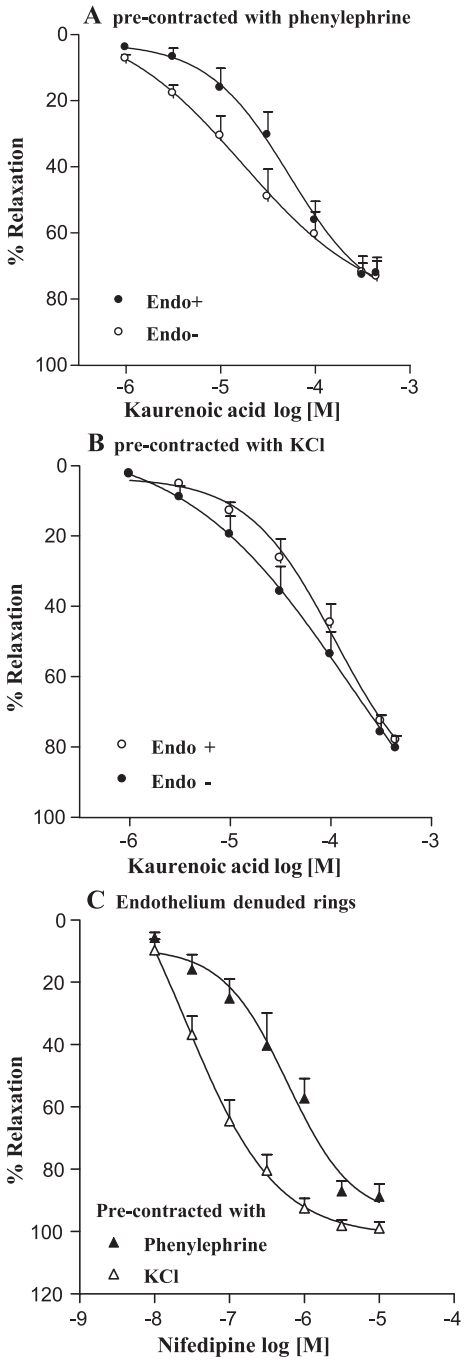

Fig. 4. Relaxation responses induced by kaurenoic acid and nifedipine on rat aortic rings. Kaurenoic acid-induced relaxation was studied on endothelium-intact (Endo+) and endothelium-denuded (Endo −) rat aortic rings submaximally pre-contracted with either phenylephrine (A) or KCl (B). Nifedipine-induced relaxation was studied on endothelium denuded aortic rings pre-contracted with both agonists (C). Steady tension was evoked by phenylephrine or KCl and then kaurenoic acid (1–450 μM) or nifedipine (10^{-8}–10^{-6} M) were added cumulatively.

Tirapelli et al., 2002) and relaxant (Tirapelli et al., 2003) effects on vascular smooth muscle preparations.

In the present study, the diterpene concentration-dependently reduced the contractions induced by phenylephrine or KCl in endothelium-intact and -denuded aortic rings. In addition, the diterpene relaxed intact and denuded rings pre-contracted with either phenylephrine or KCl. The pD_2 values as well as the percentage of relaxation induced by nifedipine on denuded rings pre-contracted with phenyleph-

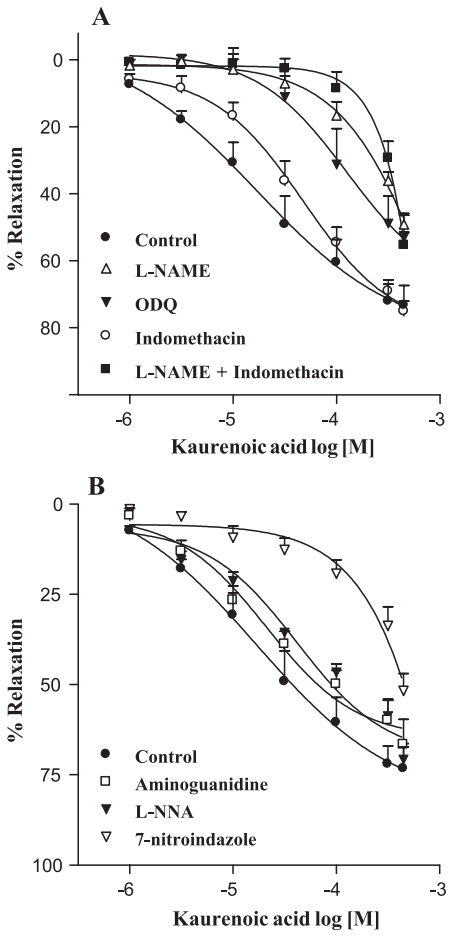

Fig. 5. Relaxation responses induced by kaurenoic acid on denuded rat aortic rings pre-contracted with phenylephrine in the presence of L-NAME (100 μM), ODQ (1 μM), indomethacin (10 μM) (A), aminoguanidine (100 μM), 7-nitroindazole (100 μM) and L-NNA (100 μM) (B). The rings were pre-incubated with the drugs for 30 min. Steady tension was evoked by phenylephrine and then kaurenoic acid (1–450 μM) was added cumulatively.

Table 3
Effect of L-NAME, ODQ, indomethacin, aminoguanidine, 7-nitroindazole and L-NNA on kaurenoic acid-induced relaxant responses of denuded rat aortic rings pre-contracted with phenylephrine

Groups	E_{max} (% relaxation)	pD_2
Control	73.18 ± 5.91	4.57 ± 0.20
L-NAME 100 μM	49.12 ± 3.26[a]	3.78 ± 0.09[a]
ODQ 1 μM	53.10 ± 6.72[a]	4.00 ± 0.11[a]
Indomethacin 10 μM	75.06 ± 3.02	4.43 ± 0.10
L-NAME + indomethacin	55.29 ± 3.86[a]	3.20 ± 0.11[a]
Aminoguanidine 100 μM	66.63 ± 6.98	4.67 ± 0.18
7-Nitroindazole 100 μM	51.74 ± 4.76[a]	3.53 ± 0.17[a]
L-NNA 100 μM	70.83 ± 4.48	4.50 ± 0.16

Values are means ± S.E.M., $n = 6$–8 experiments.
[a] One-way ANOVA: compared to control group (ANOVA followed by Dunnet's multiple comparison test, $P < 0.05$).

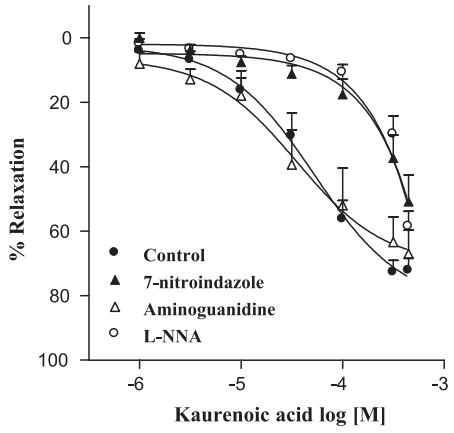

Fig. 6. Relaxation responses induced by kaurenoic acid on intact rat aortic rings pre-contracted with phenylephrine in the presence of aminoguanidine (100 μM), 7-nitroindazole (100 μM) and L-NNA (100 μM). The rings were pre-incubated with the drugs for 30 min. Steady tension was evoked by phenylephrine and then kaurenoic acid (1–450 μM) was added cumulatively.

rine or KCl were higher when compared to those found for kaurenoic acid, indicating that nifedipine is more potent than kaurenoic acid at inhibiting phenylephrine and KCl pre-contracted rings.

Another aspect investigated in this study was whether kaurenoic acid-induced vasorelaxation was related to inhibition of Ca^{2+} influx from the extracellular medium. We noted that kaurenoic acid relaxed preparations pre-contracted with KCl or phenylephrine. It also inhibited the development of contractions induced by these agonists. Finally, $CaCl_2$-induced contraction in Ca^{2+}-free medium containing KCl was inhibited concentration-dependently by kaurenoic acid. Taken together, these results support the notion that kaurenoic acid can block Ca^{2+} influx through Ca^{2+} channels presented in the vascular smooth muscle cells. However, this behaviour does not rule out the possibility that kaurenoic acid reduces the sensitivity of the contractile filaments to Ca^{2+}. Contractions of rat aortic rings induced by KCl rely almost exclusively on Ca^{2+} influx through activation of voltage-sensitive channels (Hudgins and Weiss, 1968), whereas 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., 2001a,b). Since kaurenoic acid relaxed aortic rings pre-contracted with both agonists with similar E_{max} and pD_2 values, it could be suggested that the diterpene blocks Ca^{2+} influx through interference with both voltage- and receptor-operated channels.

Table 4
Effect of aminoguanidine, 7-nitroindazole and L-NNA on kaurenoic acid-induced relaxant responses of endothelium-intact rat aortic rings pre-contracted with phenylephrine

Groups	E_{max} (% relaxation)	pD_2
Control	72.27 ± 3.79	4.29 ± 0.18
Aminoguanidine 100 μM	67.05 ± 7.33	4.35 ± 0.19
7-Nitroindazole 100 μM	50.84 ± 8.22[a]	3.03 ± 0.19[a]
L-NNA 100 μM	61.56 ± 4.70	3.13 ± 0.16[a]

Values are means ± S.E.M., $n = 5–7$ experiments.
[a] One-way ANOVA: compared to control group (ANOVA followed by Dunnet's multiple comparison test, $P < 0.05$).

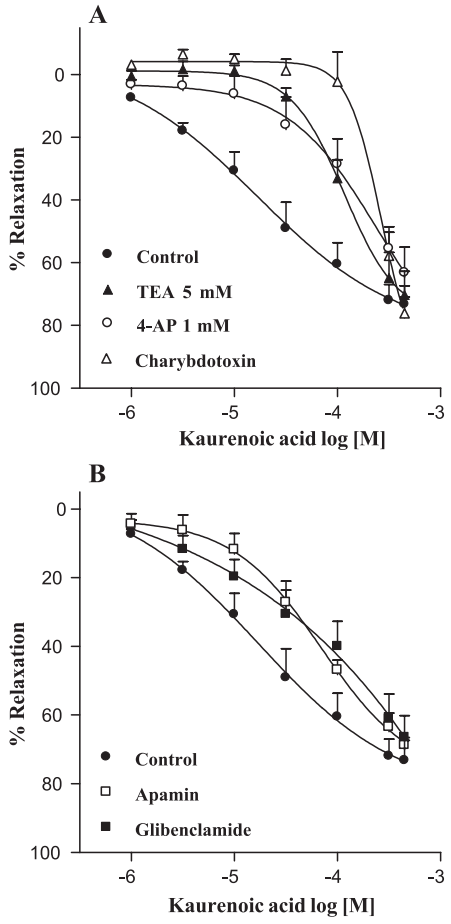

Fig. 7. Relaxation responses induced by kaurenoic acid on denuded rat aortic rings pre-contracted with phenylephrine in the presence of tetraethylammonium (5 mM), 4-amynopiridine (1 mM), charybdotoxin (0.1 μM) (A), apamin (1 μM) or glibenclamide (3 μM) (B). The rings were pre-incubated with the drugs for 30 min. Steady tension was evoked by phenylephrine and then kaurenoic acid (1–450 μM) was added cumulatively.

Table 5
Effects of tetraethylammonium, charybdotoxin, 4-amynopiridine, apamin and glibenclamide on kaurenoic acid-induced relaxant responses of denuded rat aortic rings pre-contracted with phenylephrine

Groups	E_{max} (% relaxation)	pD_2
Control	73.18 ± 5.91	4.57 ± 0.20
Tetraethylammonium 5 mM	70.32 ± 7.56	3.97 ± 0.06[a]
Charybdotoxin 0.1 μM	76.17 ± 5.24	3.65 ± 0.09[a]
4-Amynopiridine 1 mM	63.24 ± 8.21	3.80 ± 0.22[a]
Apamin 1 μM	68.80 ± 2.23	4.21 ± 0.11
Glibenclamide 3 μM	66.51 ± 6.30	4.25 ± 0.25

Values are means ± S.E.M., $n = 5–7$ experiments.
[a] Compared to control group (ANOVA followed by Dunnet's multiple comparison test, $P < 0.05$).

The present results show that, in the presence of kaurenoic acid, there was a rightward shift in the concentration–response curves for $CaCl_2$ with a decrease in the E_{max} values, indicating that the diterpene behaves as a non-competitive calcium antagonist.

The influence of kaurenoic acid in Ca^{2+} release from intracellular stores sensitive to phenylephrine and caffeine was also analysed. The diterpene did not alter the contraction induced by phenylephrine, which stimulates [$InsP_3$]-dependent Ca^{2+} release from intracellular stores (Eckert et al., 2000). Similarly, caffeine-induced contraction, which releases Ca^{2+} from intracellular stores by an [$InsP_3$]-independent mechanism (Leitjen and van Breemen, 1984), was not altered. Thus, it seems unlikely that the vascular effects of kaurenoic acid involve a reduction in Ca^{2+} release from intracellular stores sensitive to phenylephrine and caffeine. This result does not rule out the possibility that KA interferes with the sarco-endoplasmatic reticulum Ca^{2+}-ATPases. Further studies should be performed to clarify this point.

Vasodilator cyclo-oxygenase product(s) of non-endothelial origin has been described to induce endothelium-independent relaxation (Cherry et al., 1982; Förstermann et al., 1986; Ritter et al., 1989). Indomethacin, a non-selective cyclo-oxygenase inhibitor, was used to evaluate the involvement of vasodilator prostanoids in kaurenoic acid-induced relaxation. The cyclo-oxygenase inhibitor was not found to affect the relaxation caused by kaurenoic acid. Therefore, it appears that cyclooxygenase pathways do not play an appreciable role in mediating kaurenoic acid effects.

Nitric oxide, enzymatically synthesized from the amino acid L-arginine, was first discovered in the vascular endothelium (Furchgott and Zawadzki, 1980; Moncada et al., 1989). In early 1990s, Schini and Vanhoutte (1991) reported that L-arginine evokes both endothelium-dependent and -independent relaxation in L-arginine depleted rat aorta, demonstrating that the vascular smooth muscle also possesses biochemical pathways converting L-arginine to NO. In our study, pretreatment of denuded rings with L-NAME was associated with a rightward displacement of the curve and a reduction of kaurenoic acid-induced relaxation, further indicating the participation of NO in the vasorelaxant effects of kaurenoic acid. The combination of L-NAME and indomethacin showed no further suppression than that observed with L-NAME alone confirming that NO, but not vasodilator prostanoids, is involved in the relaxant response induced by kaurenoic acid.

There is now consistent evidence that NO produces vasorelaxant response in vascular smooth muscle through cGMP-dependent mechanism (Moncada et al., 1989; Robertson et al., 1993). Thus, we sought to determine the possible requirement of cGMP pathway in the vasorelaxant action of kaurenoic acid. The selective inhibitor of guanylyl cyclase enzyme, ODQ (Garthwaite et al., 1995), inhibited the endothelium-independent vasorelaxant action of kaurenoic acid. Such findings confirm the involvement of the NO–cGMP pathway in kaurenoic acid-mediated vasorelaxant responses.

The NO synthase is an enzyme that occurs in three major isoforms: endothelial, neuronal and inducible (Förstermann et al., 1994). Aminoguanidine, a selective inhibitor of inducible NO synthase, did not influence the relaxation induced by kaurenoic acid. Similarly, L-NNA, an inhibitor of NO synthase, had no effect on kaurenoic acid-induced relaxation. Conversely, 7-nitroindazole, the selective inhibitor of the neuronal isoform, produced rightward displacement of the curve and a reduction of kaurenoic acid-induced relaxation. This data suggests that the activation of neuronal NO synthase play a role in the vasorelaxant effects of kaurenoic acid.

To further analyse possible overlapping mechanisms in the relaxant effect of kaurenoic acid, the NO synthase inhibitors were also tested in the rings with endothelium. The participation of inducible NO synthase in the relaxant response induced by kaurenoic acid was discarded since aminoguanidine did not alter this response. On the other hand, L-NNA and 7-nitroindazole displaced the curves for the diterpene to the right, indicating that the endothelial and neuronal isoforms take part in the relaxant response induced by kaurenoic acid, when the endothelium is present.

The opening of K^+ channels in the cell membrane of smooth muscle cells in arteries increases K^+ efflux causing membrane potential hyperpolarization, which leads to vasodilation (Nelson and Quayle, 1995). Tetraethylammonium, a non-selective blocker of K^+ channels, produced a displacement of the concentration–response curve for kaurenoic acid to the right further indicating the participation of K^+ channels in the vasorelaxant action induced by the diterpene. Vascular smooth muscle cells express different types of K^+ channels (Kuriyama et al., 1995). Agents that block these channels are useful tools for exploring the role of a particular K^+ channel. Apamin, the selective blocker of the low conductance Ca^{2+}-activated channels, and glibenclamide, which acts as a selective blocker of ATP-sensitive K^+ channels, did not produce any significant effect on kaurenoic acid-induced relaxation. The present results obtained with rat aorta are in contrast to the observation that glibenclamide partly attenuated the relaxant effect of kaurenoic acid on rat uterus (De Alencar Cunha et al., 2003). The lack of effect of glibenclamide on the relaxation induced by kaurenoic acid on aortic rings could be due to a variation in the characteristics of the glibenclamide-sensitive K^+ channels in different tissues and in their contribution to the membrane potential in the different tissues.

Charybdotoxin, a non selective blocker of large and intermediate conductance Ca^{2+}-activated K^+ channels and of some voltage-dependent K^+channels, produced a rightward displacement of the concentration–response curve for kaurenoic acid, indicating that the opening of these channels are required for the vasorelaxant effect of kaurenoic acid. Similarly, 4-amynopiridine, a selective blocker of the voltage-dependent K^+ channels, affected kaurenoic acid-induced relaxation, indicating that the activation of voltage-dependent K^+ channels also plays a role in kaurenoic acid-induced relaxation.

Recently, it was demonstrated that the antihypertensive action of stevioside, a glycoside isolated from the leaves of *Stevia rebaudiana*, is related to its ability to reduce extracellular Ca^{2+} influx (Lee et al., 2001a,b). Clinically, Ca^{2+} antagonists are used for the treatment of hypertension due to their ability to induce smooth muscle relaxation. It is possible to suggest, considering its vascular effects, that kaurenoic acid is a potential agent that could exert antihypertensive action in vivo.

From our data, it is possible to propose that kaurenoic acid, as well as the diterpene jatrophone (Duarte et al., 1992), exerts its vasodilatory effects by acting on multiple sites of action. Kaurenoic acid blocks extracellular Ca^{2+} influx by interacting with both voltage- and receptor-operated channels. In addition, its action also involves the stimulation of neuronal NO synthase and activation of the NO–cGMP pathway, which, in turn, could be responsible for the opening of K^+ channels. The diterpene also stimulates the production of NO from endothelial cells.

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