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Pimaradienoic acid inhibits vascular contraction and induces hypotension in normotensive rats

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Abstract

The present investigation was designed to investigate the effect of the diterpene *ent*-pimara-8(14),15-dien-19-oic acid (pimaradienoic acid, PA) on smooth muscle extracellular Ca^{2+} influx. To this end, the effect of PA on phenylephrine- and KCl-induced increases in cytosolic calcium concentration ($[Ca^{2+}]_c$), measured by the variation in the ratio of fluorescence intensities (R340/380 nm) of Fura-2, was analysed. Whether bolus injection of PA could induce hypotensive responses in conscious normotensive rats was also evaluated. PA inhibited the contraction induced by phenylephrine (0.03 or 10 μmol L^{-1}) and KCl (30 or 90 mmol L^{-1}) in endothelium-denuded rat aortic rings in a concentration dependent manner. Pre-treatment with PA (10, 100, 200 μmol L^{-1}) attenuated the contraction induced by $CaCl_2$ (0.5 nmol L^{-1} or 2.5 mmol L^{-1}) in denuded rat aorta exposed to Ca^{2+}-free medium containing phenylephrine (0.1 μmol L^{-1}) or KCl (30 mmol L^{-1}). Interestingly, the inhibitory effect displayed by PA on $CaCl_2$-induced contraction was more pronounced when KCl was used as the stimulant. Phenylephrine- and KCl-induced increases in $[Ca^{2+}]_c$ were inhibited by PA. Similarly, verapamil, a Ca^{2+}-channel blocker, also inhibited the increase in $[Ca^{2+}]_c$ induced by either phenylephrine or KCl. Finally, bolus injection of PA (1–15 mg kg^{-1}) produced a dose-dependent decrease in mean arterial pressure in conscious normotensive rats. The results provide the first direct evidence that PA reduces vascular contractility by reducing extracellular Ca^{2+} influx through smooth muscle cellular membrane, a mechanism that could mediate the hypotensive response induced by this diterpene in normotensive rats.

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Introduction

It is now well established that pimarane-type diterpenes display inhibitory activity on smooth muscle contraction. Ohashi et al (2000) isolated four isopimarane-type diterpenes from the leaves of *Orthosiphon aristatus*: neo-orthosiphol A, neo-orthosiphol B, orthosiphol A and orthosiphol B. These pimaranes exhibited concentration-dependent suppression of contractions induced by KCl in endothelium-denuded rat thoracic aorta.

More recently, results from our laboratory showed that *ent*-pimara-8(14),15-dien-19-oic acid (pimaradienoic acid, PA), isolated from the roots of *Viguiera arenaria* (Asteraceae), significantly inhibited phenylephrine- and KCl-induced contraction in isolated rat carotid rings (Ambrosio et al 2002). These results suggested that the effect elicited by PA could be related to a reduction in the extracellular Ca^{2+} influx since it inhibited KCl-induced contraction, which is mainly dependent on extracellular Ca^{2+} mobilization (Hudgins & Weiss 1968). Following the initial report of a putative role of PA as a Ca^{2+}-channel blocker, the mechanisms underlying its inhibitory activity on vascular smooth muscle contractility were pursued. The diterpene was reported to reduce phenylephrine-induced contraction in endothelium-intact or -denuded rat aortic rings in a concentration-dependent manner (Tirapelli et al 2004a). Moreover, we found that pre-treatment with PA attenuated $CaCl_2$-induced contraction. Finally, the diterpene relaxed preparations pre-contracted with KCl or phenylephrine. Taken together, these results provided functional evidence that PA could block Ca^{2+} influx through interference with both voltage- and receptor-operated channels (Tirapelli et al 2004a). More recently, we described that PA is more effective than kaurenoic

acid, another diterpene that also inhibits vascular contraction (Tirapelli et al 2002, 2003, 2004b), at inhibiting KCl- and $CaCl_2$-induced contraction (Tirapelli et al 2005). However, although we had functional evidence that PA could block extracellular Ca^{2+} influx, no direct measurement of the effects of PA on cytosolic calcium was performed.

Clinically, Ca^{2+} antagonists are used for the treatment of hypertension due to their ability to induce smooth muscle relaxation. The leaves of *Orthosiphon aristatus* (Lamiaceae), which have been prescribed in traditional Indonesian medicine for the treatment of hypertension (Riswan & Sangat-Roemantyo 1991), contain isopimarane-type diterpenes, which were reported to induce inhibitory action on vascular smooth muscle contraction (Ohashi et al 2000). Considering this observation and the vascular effects of PA, we hypothesized that PA could exert hypotensive action in-vivo.

Based on the above observations, the present study was designed to investigate the effect of PA on smooth muscle extracellular Ca^{2+} influx. With this purpose, we analysed the effect of PA on phenylephrine- and KCl-induced contraction of isolated vessels and on the increase in cytosolic calcium concentration ($[Ca^{2+}]_c$) induced by these vasoactive agents. We also aimed to evaluate whether bolus injection of PA could induce hypotensive responses in conscious normotensive rats.

Material and Methods

Procedure for isolation of PA

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

Animals

Male Wistar rats were housed under standard laboratory conditions (12-h light–dark cycle at 24°C) with free access to food and water. The housing conditions and experimental protocols were in accordance with the Ethical Animal Committee of the Campus of Ribeirão Preto (University of São Paulo).

Vessel ring preparation

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, Barcelona, Spain) to measure tension in the vessels. The rings were placed in a 5-mL organ chamber containing Krebs solution gassed with 95% O_2 and 5% CO_2, and maintained at 37°C. The composition of Krebs solution was as follows (mmolL^{-1}): 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 were then allowed to equilibrate for 60 min with the bath fluid being changed every 15–20 min. 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 μmolL^{-1}) in the presence of contractile tone induced by phenylephrine (0.1 μmolL^{-1}). The rings were discarded if there was any degree of relaxation.

Effect of PA on KCl-, phenylephrine- or $CaCl_2$-induced contraction on isolated aortic rings

Endothelium-denuded rings were contracted with KCl (30 or 90 mmolL^{-1}) (control) and then washed out and pre-incubated with PA (10, 100 or 200 μmolL^{-1}) for 30 min. Subsequently, a new stimulation was performed with KCl (30 or 90 mmolL^{-1}). In another set of experiments the effect of PA (10, 100 or 200 μmolL^{-1}) was evaluated on phenylephrine-induced contraction (30 nmolL^{-1} or 10 μmolL^{-1}) as described for KCl. The concentrations of KCl and phenylephrine correspond to the EC50 (concentration that elicits 50% of the maximum effect) and the E_{max} (concentration that induces the maximum effect of the agonist) as previously determined (Tirapelli et al 2004b). The stimulation with KCl or phenylephrine 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 diterpene served as time controls.

The effects of PA on $CaCl_2$-induced contractions were assessed as previously described (Tirapelli et al 2004a). Briefly, endothelium-denuded rings were first contracted with phenylephrine (0.1 μmolL^{-1}) to deplete the intracellular Ca^{2+} stores in Ca^{2+}-free solution (~90 min) containing EGTA (1 mmolL^{-1}) and then rinsed in Ca^{2+}-free solution (without EGTA) containing KCl (30 mmolL^{-1}) or phenylephrine (0.1 μmolL^{-1}). The contractions induced by $CaCl_2$ (0.5 nmol L^{-1} or 2.5 mmolL^{-1}) were obtained in the absence of (control group) or after a 30-min incubation period with PA (10, 100 or 200 μmolL^{-1}).

Isolation of single smooth muscle cells and measurement of $[Ca^{2+}]c$

Briefly, the aortas were dissected and opened longitudinally. Endothelial cells were removed and smooth muscle cells were isolated by gentle rake friction of the aorta (no proteases were used). The isolation procedure was performed in Hanks' solution with the following composition (in mmolL^{-1}): NaCl 140; KCl 5.4; $K_2H_2PO_4$ 0.44; NaH_2PO_4 0.42; $CaCl_2$ 0.1; $NaHCO_3$ 4.17; glucose 5.5; EDTA 0.03; HEPES 5.0. The resultant cell solution was centrifuged at 200 *g* and suspended in Hanks' solution. The cells were placed on glass coverslips and kept in a humidified 37°C incubator gassed with 5% CO_2

for 4 h. Then, the cells were incubated with 5 μmol L^{-1} Fura-2AM in 1 mg mL^{-1} bovine serum albumin for 40 min at 37°C and then washed for 20 min. Dishes containing Fura-2AM-loaded cells were placed in a chamber mounted on a Nikon inverted microscope. When the Fura-2AM crosses the cytoplasmic membrane, the radical acetomethoxy (AM) is metabolized by esterases, so the fluorescent probe Fura-2 cannot cross another membrane. Thus, this measure reflects only the $[Ca^{2+}]c$. The $[Ca^{2+}]c$ was measured by using a high temporal resolution microfluorimeter system as previously described (Williams et al 1985; Yagi et al 1988). Briefly, the system comprises an inverted microscope (Nikon UVF 40×) coupled to a specially designed light path and a photomultiplier tube (Delta Ran-Photo Technology International, South Brunswick, NJ, USA) spectrophotometer and filter control unit (PTI-mod; RMF, Toronto, Canada). A spinning filter wheel with 340- and 380-nm transmissive segments controlled the excitation wavelengths. Typically, the filter wheel was operated at 135 rev s^{-1}, and fluorescence was collected by an IBM PC with Felix software for further analysis. An image mask was used to exclude the field beyond the cell, thereby reducing the background contribution of the field not occupied by the cell to the overall signal. The Fura-2 ratio, corrected for background fluorescence, was converted to $[Ca^{2+}]c$ by the ratio of R340/380 nm in which Fura-2 fluorescence was measured at 510 nm. Experiments were performed at room temperature (20–25°C).

Effect of PA on the phenylephrine or KCl-induced $[Ca^{2+}]c$ increase

The measurements of phenylephrine- or KCl-induced $[Ca^{2+}]c$ increase were performed in the absence (control) or in the presence of PA. After perfusion of the isolated cells with Krebs solution for 10 min, the solution was replaced with another containing PA (100 or 200 μmol L^{-1}). After a 10-min period of incubation with PA, the cells were exposed to phenylephrine (10 μmol L^{-1}) for 10 min. The same protocol was used to test the effect of PA on the increase in $[Ca^{2+}]c$ induced by KCl (30 mmol L^{-1}). The effect of the calcium-channel blocker verapamil (1 μmol L^{-1}, 10 min) on phenylephrine- or KCl-induced $[Ca^{2+}]c$ increase was also analysed for comparison.

Effect of PA on mean arterial pressure (MAP) in conscious rats

On the day before the experiments the rats were anaesthetized with tribromoethanol (2.5% i.p.) and a catheter (a 4-cm segment of PE-10 heat-bound to a 13-cm segment of PE-50; Clay Adams, Parsippany, NJ, USA) was inserted into the abdominal aorta through the femoral artery to record blood pressure. A second catheter was implanted into the femoral vein for bolus intravenous administration of PA. Both catheters were tunnelled under the skin and exteriorized at the animal's dorsum.

During the experiment, the freely moving rats were kept in individual cages and MAP was recorded using a Power Lab 2/20 (ADInstruments, Sydney, Australia) connected to a signal acquisition board (Chart 4.0) and computer processed. Dose–response curves for PA were obtained by bolus intravenous injection of the diterpene (1–15 mg kg^{-1}). The effect of verapamil (10–1000 μg kg^{-1}) on blood pressure was also evaluated. Blood pressure responses were calculated based on the average mean blood pressure calculated at the response's plateau.

Drugs

The following drugs were used: phenylephrine hydrochloride, acetylcholine hydrochloride, verapamil (Sigma, St Louis, MO, USA). Fura-2 AM was obtained from Molecular Probes (Eugene, OR, USA); potassium chloride and calcium chloride were from Synth (São Paulo, Brazil). For the in-vitro experiments, PA was prepared as stock solutions in dimethylsulfoxide (DMSO). The other drugs were dissolved in distilled water. The concentration of DMSO did not exceed 0.5%, which was shown to have no effect on the basal tonus of the preparations or on the agonist-mediated contraction or Ca^{2+} influx. For the in-vivo experiments, PA was dissolved in Tween 80 (2%), brought to the chosen volume with sterile isotonic saline. Bolus injection of the solvent did not affect MAP.

Statistical analysis

Results are expressed as means ± s.e.m. Statistical analyses were performed using Student's *t*-test or one-way analysis of variance (followed by Bonferroni's multiple comparison text) as indicated. The significance level considered in all tests was 0.05.

Results

Solvent test

Since DMSO was used as solvent for stock solutions of PA, the effects of this solvent at equivalent concentrations were assessed as time-matched controls for the protocols for the in-vitro studies (vascular reactivity and isolated cells). The assays carried out using only the solvent (concentration of DMSO did not exceed 0.5%) showed that this solvent did not affect the vascular contractility induced by the agonists or the $[Ca^{2+}]c$ (data not shown). We also found that Tween 80 at 2% did not affect the basal MAP (MAP before injection of Tween: 102.3 ± 1.4 mmHg; MAP after injection of Tween: 103.5 ± 1.1 mmHg, n = 3) (Student's *t*-test).

Effect of PA on contractile response induced by phenylephrine, KCl or $CaCl_2$

The chemical structure of PA is represented in Figure 1. Pre-treatment with PA attenuated phenylephrine-induced contraction in endothelium-denuded aortic rings (Table 1). The contraction induced by phenylephrine at 30 nmol L^{-1} was significantly reduced in the presence of PA at 10 and 100 μmol L^{-1}, and completely abolished in the presence of PA at 200 μmol L^{-1}. However, when phenylephrine was used at 10 μmol L^{-1}, the inhibition on the contraction displayed by PA was observed when the diterpene was used at 100 and

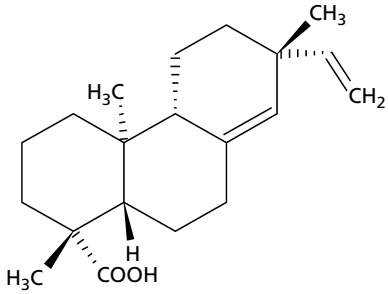

Figure 1 Chemical structure of *ent*-pimara-8(14),15-dien-19-oic acid (pimaradienoic acid).

200 μmol L^{-1}, but not at 10 μmol L^{-1}. PA at 10, 100 and 200 μmol L^{-1} inhibited the contraction induced by both concentrations of KCl (30 and 90 mmol L^{-1}).

Pre-treatment with PA attenuated $CaCl_2$-induced contraction of denuded rat aorta exposed to Ca^{2+}-free medium containing phenylephrine or KCl (Table 2). It is interesting to note that the inhibitory effect displayed by PA on $CaCl_2$-induced contraction was more pronounced when KCl was used as the stimulant.

Effect of PA on the phenylephrine or KCl-induced [Ca^{2+}]c increase

As shown in Figure 2, the phenylephrine-induced increase in [Ca^{2+}]c (measured as the Fura-2 ratio, R340/380) was inhibited by PA at 200 μmol L^{-1}. On the other hand, the diterpene inhibited the KCl-induced increase in [Ca^{2+}]c at 100 or 200 μmol L^{-1}. The Ca^{2+}-channel blocker verapamil also inhibited the increase in [Ca^{2+}]c induced by either phenylephrine or KCl.

Effect of PA on mean arterial pressure (MAP) in conscious rats

The maximal variation in MAP induced by PA or verapamil in conscious normotensive rats is presented in Figure 3. The basal value of MAP was 98 ± 1.6 mmHg (n = 4). Baseline MAP remained significantly unchanged following successive intravenous injections of PA or verapamil. Bolus injection of PA (1 to 15 mg kg^{-1}) produced a dose-dependent decrease in MAP in conscious normotensive rats. After all doses of PA tested, pre-dose values of MAP were fully recovered within the first minute after PA injection. Verapamil (10–1000 μg kg^{-1}) was found to be more potent than PA in inducing a decrease in MAP.

Table 1 Effect of pimaradienoic acid (PA) on the contraction (g) induced by phenylephrine and KCl in endothelium-denuded aortic rings

PA (μmol L^{-1})	Vasoactive agent			
	30 nmol L^{-1} Phenylephrine	10 μmol L^{-1} Phenylephrine	30 mmol L^{-1} KCl	90 mmol L^{-1} KCl
0	1.6 ± 0.1	2.2 ± 0.1	1.18 ± 0.1	1.83 ± 0.11
10	0.62 ± 0.15[a]	1.96 ± 0.17	0.65 ± 0.07[a]	0.9 ± 0.2[a]
100	0.25 ± 0.1[a,b]	0.84 ± 0.18[a,b]	0.08 ± 0.02[a,b]	0.12 ± 0.02[a,b]
200	0.11 ± 0.01[a,b]	0.1 ± 0.01[a,b,c]	0.07 ± 0.01[a,b]	0.1 ± 0.01[a,b]

Values are means ± s.e.m., n = 5–9 experiments. [a]$P < 0.05$, significantly different compared with the respective control group. [b]$P < 0.05$, significantly different compared with 10 μmol L^{-1} PA. [c]$P < 0.05$, significantly different compared with 100 μmol L^{-1} PA (analysis of variance followed by Bonferroni's multiple comparison test).

Table 2 Effect of pimaradienoic acid (PA) on $CaCl_2$-induced contractile response in Ca^{2+}-free solution containing 0.1 μmol L^{-1} phenylephrine or 30 mmol L^{-1} KCl

PA (μmol L^{-1})	Stimulant			
	0.1 μmol L^{-1} Phenylephrine		30 mmol L^{-1} KCl	
	0.5 nmol L^{-1} $CaCl_2$	2.5 mmol L^{-1} $CaCl_2$	0.5 nmol L^{-1} $CaCl_2$	2.5 mmol L^{-1} $CaCl_2$
0	0.91 ± 0.11	1.22 ± 0.11	0.9 ± 0.1	1.28 ± 0.04
10	0.76 ± 0.1	0.89 ± 0.1[a]	0.22 ± 0.04[a]	0.3 ± 0.03[a]
100	0.15 ± 0.04[a,b]	0.21 ± 0.06[a,b]	0.06 ± 0.02[a,b]	0.16 ± 0.02[a,b]
200	0.04 ± 0.01[a,b]	0.023 ± 0.01[a,b,c]	0.03 ± 0.01[a,b]	0.04 ± 0.01[a,b]

Values are means ± s.e.m., n = 4–5 experiments. [a]$P < 0.05$, significantly different compared with the respective control group. [b]$P < 0.05$, significantly different compared with 10 μmol L^{-1} PA. [c]$P < 0.05$, significantly different compared with 100 μmol L^{-1} PA (analysis of variance followed by Bonferroni's multiple comparison test).

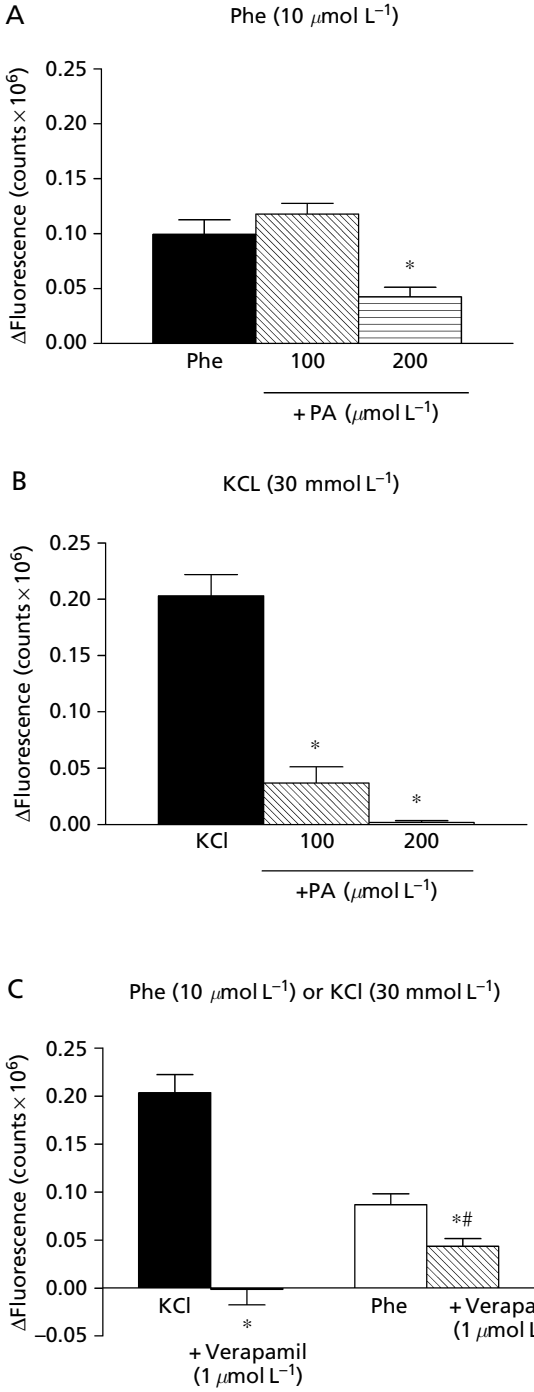

Figure 2 Effect of pimaradienoic acid (PA) on the variation of Fura-2 fluorescence ratio (R340/380) induced by phenylephrine (Phe) or KCl. Bars represent the fluorescence ratio of 5 μmol L^{-1} Fura-2 (R340/R380 nm) measured as a function of the ratio of the variation of the fluorescence intensity of Fura-2 (R340/R380 nm) induced by Phe or KCl in the absence (control) or after a 10-min period of incubation with PA at 100 or 200 μmol L^{-1} (A and B), or verapamil at 1 μmol L^{-1} (C). Each bar is the mean ± s.e.m. of four individual cells. $^{*}P < 0.05$, significantly different compared with control; $^{\#}P < 0.05$, significantly different compared with KCl + 1 μmol L^{-1} verapamil (analysis of variance followed by Bonferroni's multiple comparison test).

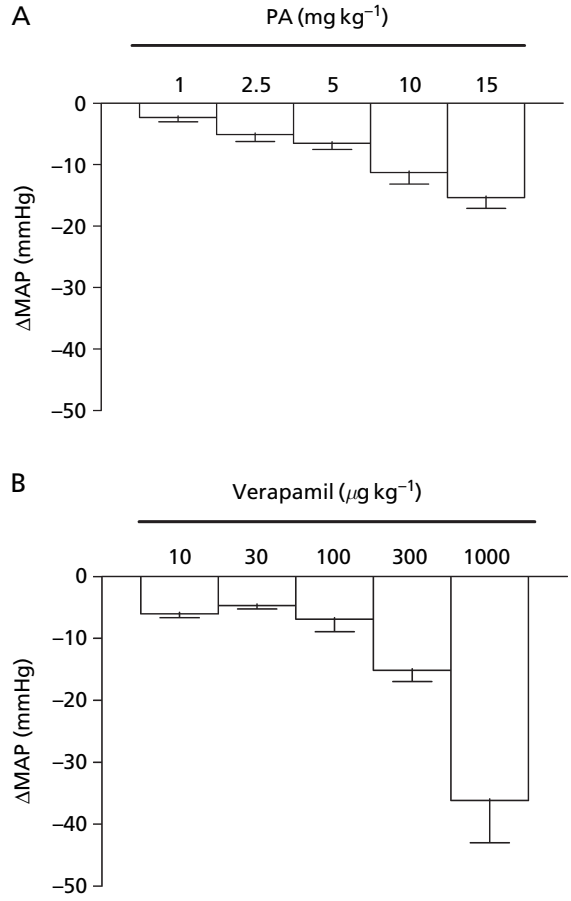

Figure 3 Maximal variation in mean arterial pressure (MAP) induced by intravenous injection of 1–15 mg kg^{-1} pimaradienoic acid (PA) or 10–1000 μg kg^{-1} verapamil in conscious normotensive rats. Each point represents the mean ± s.e.m. of four experiments for the maximal depressor response induced by PA (A) or verapamil (B).

Discussion

The present findings corroborate those of our previous study, describing that PA displays an antispasmodic effect on vascular smooth muscle (Ambrosio et al 2002; Tirapelli et al 2002, 2004a, b). Previously, we showed that PA-induced relaxation was similar in endothelium-intact and -denuded aortic rings pre-contracted with either phenylephrine or KCl. Moreover, PA attenuated the contraction induced by phenylephrine in both endothelium-intact and -denuded rings, further indicating that that the endothelium does not play a significant role in the vascular effects elicited by PA (Tirapelli et al 2004a). For this reason, the present investigation focused on the effects of PA on the smooth muscle.

It is well know that high-K^+-induced contraction in smooth muscle is mediated by cell membrane depolarization and an increase in Ca^{2+} influx through voltage-gated Ca^{2+} channels (Hudgins & Weiss 1968; Godfraind & Kaba 1969; Somlyo & Somlyo 1994). KCl-induced contraction is strongly inhibited by agents blocking the Ca^{2+} channels,

including verapamil and nifedipine (Karaki et al 1997). On the other hand, phenylephrine-induced contraction is 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). The present finding, which shows that PA inhibited both KCl- and phenylephrine-induced contraction in the isolated aortic rings, suggest that the diterpene blocks Ca^{2+} influx through interference with both voltage- and receptor-operated channels. The inhibitory action displayed by PA was greater in KCl-induced contraction than phenylephrine-induced contraction. We previously demonstrated that PA is more potent in relaxing aortic rings pre-contracted with KCl than phenylephrine (Tirapelli et al 2004a). This observation indicates that PA is more selective at inhibiting extracellular Ca^{2+} influx through voltage-operated channels. Moreover, it was found that PA produced a more accentuated reduction in the contraction induced by $CaCl_2$ in Ca^{2+}-free solution containing KCl than in Ca^{2+}-free solution containing phenylephrine. This observation strengthened our initial hypothesis that PA is more selective at inhibiting extracellular Ca^{2+} influx through voltage-operated channels, which is in accordance with previous findings from our laboratory (Tirapelli et al 2004a).

Two types of stimulants are widely used in vascular smooth muscle to increase the cytosolic Ca^{2+} level: high-K^+-induced membrane depolarization and the activation of the receptor by contractile agonists such as phenylephrine (Karaki et al 1997). An increase in the cytosolic Ca^{2+} concentration is the major trigger for smooth muscle contraction because it leads to the formation of the complex Ca^{2+}–calmodulin, whose process culminates in muscle contraction (Araujo & Bendhack 2003). In the present study, the $[Ca^{2+}]c$ was measured by the variation in the ratio of Fura-2 fluorescence, which specifically binds to cytosolic Ca^{2+} (Williams et al 1985). The results obtained with Fura-2 suggest that PA exerts its inhibitory action on vascular contractility by inhibiting the increase in the $[Ca^{2+}]c$ induced by vasoactive agents. The more pronounced effect of PA in inhibiting the KCl-induced increase in the $[Ca^{2+}]c$ corroborates the findings obtained with the experiments for vascular reactivity in the isolated aorta. Previously, it was reported that verapamil inhibits the contraction induced by phenylephrine and KCl, as well as the increase in $[Ca^{2+}]c$ (Shimizu et al 1995). Our observations confirm this finding. The possibility that PA interferes with Ca^{2+} release from intracellular stores to reduce Ca^{2+} availability for contraction was previously investigated (Tirapelli et al 2004a). The diterpene did not alter the contraction induced by phenylephrine, which stimulates inositol triphosphate (IP_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 IP_3-independent mechanism (Leitjen & van Breemen 1984), was not altered. Thus, it seems unlikely that the reduction in $[Ca^{2+}]c$ induced by PA involves a reduction in Ca^{2+} release from intracellular stores sensitive to phenylephrine and caffeine. This result does not rule out the possibility that PA interferes with the sarco-endoplasmatic reticulum Ca^{2+}-ATPases. Further studies need to be performed to clarify this point.

With regard to the vascular smooth muscle, the main Ca^{2+} channel is the L-type (McDonald et al 1994). In this work, we have used verapamil as a Ca^{2+}-channel blocker because we could not use nifedipine, a light sensitive drug, to measure $[Ca^{2+}]c$. Interestingly, verapamil induced a greater attenuation of the increase in $[Ca^{2+}]c$ stimulated by KCl than phenylephrine, suggesting that the calcium influx induced by phenylephrine occurs through the L-type calcium channels and the receptor-operated calcium channels insensitive to verapamil. This observation corroborates previous findings that the contraction evoked by phenylephrine is less sensitive to the Ca^{2+}-channel blockers than the contraction induced by KCl (Karaki et al 1997). Moreover, our data indicate that L-type Ca^{2+} channels are involved in phenylephrine- and KCl-induced increases, since verapamil, a selective blocker for these channels, inhibited these responses. Thus, we could suggest that PA would also act on L-type channels to promote its vascular effects.

The results obtained with Fura-2 on isolated aorta smooth muscle cells and the findings on the vascular reactivity experiments allowed us to conclude that the inhibitory action displayed by PA on the smooth muscle contractility in the rat aorta is due to the decrease in $[Ca^{2+}]c$. However, this behaviour does not rule out the possibility that PA reduces the sensitivity of the contractile filaments to Ca^{2+}.

Clinically, Ca^{2+}-channel blockers are used for the treatment of hypertension because of their ability to induce smooth muscle relaxation (Sahney 2006; Triggle 2006). The leaves of *O. aristatus* (Lamiaceae) are widely used in traditional Indonesian medicine for the treatment of hypertension (Riswan & Sangat-Roemantyo 1991). These leaves contain isopimarane-type diterpenes, which were reported to induce inhibitory action on vascular smooth muscle contraction (Ohashi et al 2000). More recently, Lahlou et al (2007) showed that the diterpene Labd-8 (17)-en-15-oic acid relaxes rat aortic rings pre-contracted with KCl- and induces a dose-dependent hypotensive response in normotensive rats. Such observations suggest that the hypotensive action displayed by naturally occurring diterpenes is related to their myorelaxant activity. Since PA inhibits the vascular contraction of isolated rat aorta and the increase in $[Ca^{2+}]c$ induced by phenylephrine and KCl, we hypothesized that this diterpene could exert hypotensive action. To our knowledge, this is the first report to describe the hypotensive effect of PA in normotensive rats. The experiments on isolated vascular tissues and on isolated smooth muscle cells loaded with Fura-2 support the notion that PA-induced hypotension could be mediated by the direct vasorelaxant action of this diterpene on the vascular smooth muscle, a response that involves the blockade of extracellular Ca^{2+} influx. The vascular smooth muscle possesses biochemical pathways to produce vasodilator cyclooxygenase products (Cherry et al 1982; Förstermann et al 1986; Ritter et al 1989) and nitric oxide (NO) (Schini & Vanhoutte 1991), which are reported to induce endothelium-independent relaxation. Previously, we found that indometacin, a non-selective cyclooxygenase inhibitor, as well as L-NAME, a non-selective NO synthase inhibitor, attenuated the relaxation induced by PA in endothelium-denuded aortic rings pre-contracted with phenylephrine (Tirapelli et al 2004a). Moreover, the selective inhibitor of guanylyl cyclase enzyme, ODQ (Garthwaite et al 1995), inhibited the endothelium-independent vasorelaxant effect induced by PA. Such findings indicate that the endothelium-independent vascular effects of PA are

partly dependent on the activation of the NO–cGMP pathway and the release of metabolites derived from the arachidonic acid pathway (Tirapelli et al 2004a). Thus, the precise mechanism by which PA exerts its hypotensive action requires further investigation. Moreover, the present findings do not rule out participation of the central sympathetic outflow in the hypotensive response evoked by PA.

Conclusion

Using a combined in-vivo and in-vitro approach, the present study shows, for the first time, that intravenous treatment of conscious normotensive rats with PA lowers blood pressure, probably through an active vascular relaxation that involves the blockade of extracellular Ca^{2+} influx.

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