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Vasorelaxing effect in rat thoracic aorta caused by laurotetanine isolated from *Litsea cubeba* Persoon

WEN-YING CHEN, FENG-NIEN KO*, YANG-CHANG WU†, SHENG-TEH LU†, CHE-MING TENG*, *Foo-Yin Junior College of Nursing & Medical Technology, Kaohsiung, *Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, and †Graduate Institute of Natural Products, Kaohsiung Medical College, Kaohsiung, Taiwan*

Abstract—The pharmacological effects of laurotetanine on rat isolated thoracic aorta were examined. The contraction of aortic rings caused by high potassium (60 mM) and cumulative concentrations of calcium (0·03–3 mM) was inhibited by 3–50 μM laurotetanine in a dose-dependent manner with an IC50 value of 19·8 ± 3·6 μM (n = 6) in a 1 mM Ca^{2+} medium. The phenylephrine (3 μM)-induced contraction was also inhibited by laurotetanine. Its effect was more marked on the tonic contraction than on the phasic contraction and was not easily washed-out. On addition of laurotetanine during the tonic contraction, relaxation could also be observed. This relaxing effect was not antagonized by indomethacin (20 μM) and was still seen in denuded aorta or in the presence of nifedipine (1 μM). The caffeine (20 mM)-induced contraction was not affected by laurotetanine. cAMP and cGMP levels of aorta were not changed by laurotetanine. It is concluded that laurotetanine relaxed the rat thoracic aorta mainly by suppressing the Ca^{2+} influx through both voltage- and receptor-operated calcium channels.

Litsea cubeba Persoon is a folk medicine in traditional Chinese therapy against vomitting, flatulence and diarrhoea. The aqueous extract may prevent mosquitoe bites and inhibits *Suchistosoma japanicum* in-vitro (Kiangsu Provincial New Medical College 1977). Laurotetanine (Fig. 1) is an aporphine alkaloid isolated from *Litsea cubeba* Persoon (Tomita et al 1965). Recently, we have screened and purified some active components affecting the cardiovascular system from Chinese herbs, and we found laurotetanine caused marked vasorelaxation of rat thoracic aorta. In this paper, we describe this action and attempt to elucidate its mechanism of action.

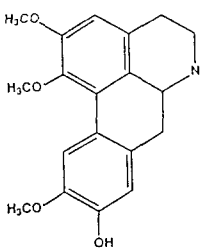

FIG. 1. Structure of (+)-laurotetanine.

Materials and methods

Mechanical response. Wistar rats of either sex, 250–300 g, were killed by a blow to the head. The thoracic aorta was isolated and excess fat and connective tissue were removed. The vessel was cut into rings of about 5 mm in length and mounted in organ baths containing 5 mL Krebs solution of the following composition (mM): NaCl 118·2, KCl 4·7, $CaCl_2$ 1·9, $MgSO_4$ 1·2, KH_2PO_4 1·2, $NaHCO_3$ 25 and glucose 11·7. The tissue-bath solution was maintained at 37°C and bubbled with 95% O_2–5% CO_2. Two stainless steel hooks were inserted into the aortic lumen, one was fixed while the other was connected to a transducer. Aortae were equilibrated in the medium for 90 min with three changes of Krebs solution and maintained under an optimal tension of 1 g before specific experimental protocols were initiated. Contractions were recorded isometrically via a force-displacement transducer connected to a Grass polygraph (Model 79D). There was no significant change in the contractility of the tissue over 5–6 h. In some experiments, the endothelium was removed by rubbing with a cotton ball. These denuded rings did not relax to acetylcholine (3 μM) after they had been contracted by phenylephrine (3 μM).

The contractile effects of calcium were studied in rings stabilized in K^+ solution without Ca^{2+}. Calcium was then added from stock dilutions to obtain the desired contractions, and the effects of each Ca^{2+} concentration were recorded. The maximal tension attained at 3 mM Ca^{2+} was considered as 100%. The high-K^+ solutions were prepared by substituting NaCl with KCl (60 mM) in an equimolar amount.

Cyclic (c) AMP and cGMP assay. The amount of cAMP and cGMP in aortic rings was assayed as described by Kauffman et al (1987). After incubation of aortic rings with dimethylsulphoxide, forskolin, sodium nitroprusside or laurotetanine for 2 min, the aortic rings were rapidly frozen in liquid nitrogen and stored at −80°C until homogenized in 0·5 mL 10% trichloroacetic acid using a Potter glass/glass homogenizer. The homogenate was centrifuged at 10000 *g* for 5 min and the supernatant was removed and extracted with 4 × 3 vol ether, and the cAMP and cGMP content was then assayed using enzyme immunoassay (EIA) kits. The precipitate was used for protein assay (Lowry et al 1951). cAMP and cGMP levels were expressed as pmol (mg protein)$^{-1}$.

Materials. (+)-Laurotetanine was isolated from *Litsea cubeba* Persoon as brown crystals of mp 113–114°C (sinter at 107°C), $[\alpha]_D^{31.5}$ +105·9° (ethanol) and identified by direct comparison (UV, IR, TLC and ^{1}H NMR) with authentic samples (Tomita et al 1965). Phenylephrine, acetylcholine, nifedipine, procaine, indomethacin, sodium nitroprusside, forskolin and caffeine were obtained from Sigma Chemical Co. cAMP and cGMP EIA kits were purchased from Cayman Chemical Co. If drugs were dissolved in dimethylsulphoxide (DMSO), the final concentration of DMSO in the bathing solution did not exceed 0·1% and had no effect on the muscle contraction.

Data analysis. The experimental results of the mechanical response were calculated as the relative percentage of control. All data were expressed as the mean ± s.e.m. Significant differences were tested using an unpaired Student's *t*-test and *P* values < 0·05 were considered significant. The IC50 values, defined as the concentration of the drug giving 50% inhibition of the maximal response obtained, were determined graphically by interpolation.

Results

Effects of laurotetanine on high K^+-induced Ca^{2+}-dependent contraction. In Ca^{2+}-free Krebs solution containing high K^+ (60

Correspondence: C. M. Teng, Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Taiwan.

mM), the cumulative addition of Ca^{2+} (0·03–3 mM) caused a stepwise increasing contraction. A maximal contraction of 1·65±0·07 g (n=24) was obtained. After pretreatment for 15 min, laurotetanine inhibited this contraction concentration-dependently (Fig. 2). The IC50 was calculated to be 19·8±3·6 μM (n=6) for a Ca^{2+} concentration of 1 mM. In normal Krebs solution, exposure of rat aorta to KCl (60 mM) caused a tonic contraction maintained over 30 min. Pretreatment with laurotetanine (100 μM) for 15 min completely inhibited the tonic contraction. On addition of laurotetanine 10 min after the addition of KCl, a relaxation was also observed (65·2±7·7%, n=6). Under the same conditions, nifedipine (1 μM) completely relaxed the tonic contraction.

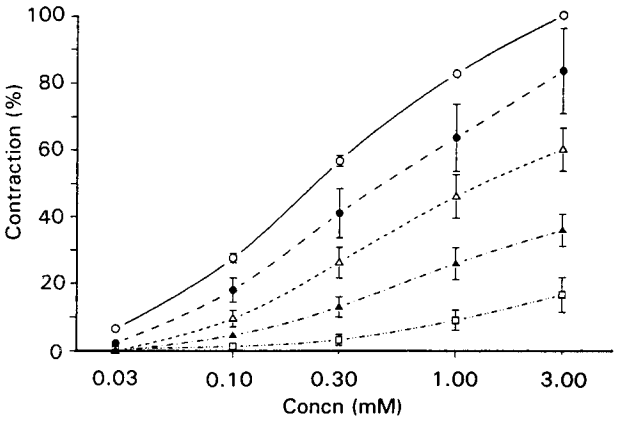

FIG. 2. Effects of laurotetanine on the Ca^{2+}-dependent contraction of rat aorta induced by high K^+ (60 mM). Aortic rings were preincubated with 0·1% DMSO (○) or various concentrations of laurotetanine (● 3, △ 10, ▲ 30, □ 50 μM) at 37°C for 15 min; cumulative concentrations of Ca^{2+} (0·03–3 mM) were then used to trigger the contraction. Each point represents the mean±s.e.m. of 5–6 experiments.

Effects of laurotetanine on phenylephrine-induced contraction. Phenylephrine (3 μM) caused a rapid phasic and then a tonic contraction maintained for at least 30 min. The response at 15 min after the addition of phenylephrine was assumed as 100% (control). A more than 70% relaxation caused by acetylcholine (3 μM) indicated that the endothelium of the aortic ring was intact. Pretreatment with laurotetanine (30–100 μM) for 15 min inhibited the phenylephrine-induced phasic and tonic contraction in a dose-dependent manner. The inhibitory effect on the tonic contraction, with an IC50 of 36·8±4·8 μM (n=6), was more potent than that on the phasic contraction. There were no obvious differences in the comparison of these inhibitory effects with those on denuded aorta, despite the potency of the high concentration of laurotetanine (100 μM) on phasic contraction being greater than on the intact preparation (Table 1). None of the inhibitory effects were easily washed-out until incubation time was prolonged to 2 h. Addition of laurotetanine 10 min after exposure to phenylephrine caused a relaxation in both intact and denuded aorta. This relaxation was not affected by indomethacin (20 μM) added 3 min before laurotetanine (80·2±6·5%, n=3) and was still seen in the presence of nifedipine (1 μM) which completely blocked KCl-induced contraction.

Effect of laurotetanine on caffeine-induced contraction. In Ca^{2+}-free Krebs solution, caffeine (20 mM) caused a rapid phasic contraction. Laurotetanine (100 μM) and nifedipine (2 μM) did not affect, while procaine (10 mM) completely abolished, the caffeine-induced phasic contraction.

Effect of laurotetanine on cAMP and cGMP formation. Forskolin (10 μM) and sodium nitroprusside (10 μM) significantly increased cAMP and cGMP levels, respectively. Neither cAMP nor cGMP levels were changed by laurotetanine (30–100 μM) (Table 2).

Discussion

Endothelium-dependent relaxation of blood vessels is produced by many agents (e.g. acetylcholine, ATP, substance P, histamine). This relaxing effect results from releasing endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI_2) (Jaffe 1985). EDRF activates soluble guanylate cyclase of the smooth muscle, and the resulting increase in cGMP levels produces relaxation (Forstermann et al 1986; Vanhoutte et al 1986; Furchgott & Vanhoutte 1989). Indomethacin abolishes the generation of PGI_2. The relaxing action of laurotetanine persisted in endothelium-denuded or intact aorta in the presence of indomethacin (20 μM). Thus, the vasorelaxation caused by laurotetanine is independent of the endothelium. In denuded aorta, a lower concentration (30 μM) of laurotetanine had weaker efficacy than in intact aorta. We suggest that phenylephrine-induced contractions are larger in denuded aorta, so the inhibitory effect could not be easily observed.

Cyclic nucleotides play an important role in relaxing vascular smooth muscle (Murad 1986). Sodium nitroprusside and forskolin are both very potent smooth muscle-relaxing agents. The former increases the cGMP level via direct activation of guanylate cyclase (Gruetter et al 1979), while the latter increases the cAMP level via direct activation of adenylate cyclase

Table 1. Effects of laurotetanine on the phasic and tonic contractions induced by phenylephrine in rat aorta.

Laurotetanine added 15 min before phenylephrine (μM)	Contraction (%)			
	Intact aorta		Denuded aorta	
	Phasic	Tonic	Phasic	Tonic
30	86.5±2·8**	68·2±9·9*	79·1±4·2**	71·6±7·3*
50	56·8±5·7**	29·6±7·7**	41·3±10·8**	27·3±12·0**
100	25·2±5·9**	7·2±4·2**	1·3±1·1**	2·2±1·4**
Laurotetanine added 10 min after phenylephrine (μM)	Relaxation (%)			
	Intact aorta		Denuded aorta	
30	46·5±7·1**		7·9±2·1**	
50	63·3±4·1**		42·2±13·9*	
100	78·9±3·9**		84·9±7·6**	

*$P<0·01$, **$P<0·001$ compared with the corresponding DMSO control. Results are mean±s.e.m.

Table 2. Effect of laurotetanine on the cAMP and cGMP formation of rat aorta.

Treatment	cAMP (pmol (mg protein)$^{-1}$)	cGMP
Control	2·26±0·19	1·27±0·11
Sodium nitroprusside (10 μM)		10·24±0·81*
Forskolin (10 μM)	11·80±3·74*	
Laurotetanine (100 μM)	2·99±0·30	1·36±0·10

After preincubation of aortic rings with DMSO (0·1%, control), sodium nitroprusside, forskolin or laurotetanine for 15 min, the reaction was stopped by immersing the tissue in liquid nitrogen. The values are expressed as the mean±s.e.m. (n=6). *$P<0·001$ compared with the corresponding control.

(Ousterhout & Sperelakis 1987). Neither cGMP nor cAMP levels were increased, suggesting that the inhibition by laurotetanine on phenylephrine or high K^+-induced contraction was not due to the increase in cyclic nucleotides.

It is widely accepted that the mechanism of phenylephrine-induced phasic contraction is by activation of α_1-adrenergic receptors and subsequent stimulation of phosphoinositide turnover to increase the concentration to inositol trisphosphate (Hashimoto et al 1986) which releases the cellular Ca^{2+} store to mediate the phasic contraction (Suematsu et al 1984). Laurotetanine inhibited the phasic contraction concentration-dependently, but not the caffeine-induced contraction. As it has been reported that caffeine could release intracellular Ca^{2+} to cause a phasic contraction in vascular smooth muscle (Saida & Van Breemen 1984), and caffeine-sensitive Ca^{2+} pools were different from phenylephrine-sensitive Ca^{2+} pools (Kanaide et al 1987), we suggest that laurotetanine may inhibit caffeine-insensitive Ca^{2+} stores.

High K^+-induced vascular smooth muscle contraction results from the increase of extracellular Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Karaki & Weiss 1979). The high K^+-induced Ca^{2+} cumulative contraction was inhibited by laurotetanine. This inhibition was more effective than that on the phasic and the tonic contraction caused by phenylephrine. Increasing Ca^{2+} concentration could partially reverse the contraction which was inhibited by laurotetanine (Fig. 2). For example, 0·03 mM Ca^{2+}-induced contraction was inhibited completely while 3 mM Ca^{2+}-induced contraction was inhibited by only 40·0±6·4% by pretreatment with 10 μM laurotetanine under the same conditions. These results indicated that laurotetanine is a voltage-dependent Ca^{2+}-channel blocker.

Extracellular Ca^{2+} entry through receptor-operated Ca^{2+} channels contributes to the tonic contraction induced by α_1-adrenoceptor agonists (Bolton 1979). Laurotetanine relaxed this tonic contraction even in the presence of nifedipine (1 μM) which blocked completely the 60 mM K^+-induced contraction. Thus laurotetanine might also possess the inhibitory property of receptor-operated Ca^{2+} channels.

In conclusion, we suggest that laurotetanine might relax the rat thoracic aorta by suppressing Ca^{2+} influx through both voltage- and receptor-dependent calcium channels. The possibility of laurotetanine affecting the intracellular events necessary for the receptor activation-contraction or ion-channel opening-contraction coupling could not be ruled out.

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