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Cardiovascular Effects of Plant Secondary Metabolites Norarmepavine, Coclaurine and Norcoclaurine

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The cardiovascular effects of (±)-norarmepavine, a benzylisoquinoline alkaloid of natural origin, have been determined on anaesthetized rats *in vivo,* on spontaneously beating atria and on aortic smooth muscle. In aorta, the effects of (±)-coclaurine and (±)-norcoclaurine, benzylisoquinolines with a related structure, were also compared.

(±)-Norarmepavine (10 mg/kg *i.v.*) decreased the mean arterial pressure and heart rate by 45% and 21%, respectively. (±)-Norarmepavine (10^{-5}–10^{-3} M) showed a negative chronotropic effect on rat-isolated atria, decreasing the spontaneous frequency by about 54%.

Aortic rings contracted with KCl 70 mM were relaxed in a concentration-dependent manner by (±)-norarmepavine, (±)-coclaurine and (±)-norcoclaurine (10^{-6}–10^{-3} M). The two earlier alkaloids exhibited an efficacy similar to verapamil, relaxing the aortic rings by 100%. (±)-Norcoclaurine exhibited a lower efficacy. These results point to the importance of methylation of these compounds. The rank order of potency was: (±)-verapamil > (±)-norarmepavine > (±)-norcoclaurine > (±)-coclaurine.

The alkaloids shifted to the right the calcium-dependent contraction curves, denoting a calcium antagonist-like effect; however, only a 10-fold increment of (±)-norcoclaurine concentration produced an equivalent effect. Our results demonstrate the hypotensive and bradycardic properties of (±)-norarmepavine. It is proposed that this alkaloid could somehow modulate calcium entry, its intracellular release or the calcium sensitivity of the cell contractile-machinery, previously postulated for coclaurine. (±)-Norcoclaurine effects reported here are not in agreement with the proposal of (±)-norcoclaurine as a calcium channel activator or β_1-adrenoceptor agonist. © **1998 John Wiley & Sons, Ltd.**

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Keywords: benzylisoquinolines; calcium antagonist; blood pressure; heart rate; rat aorta; vascular relaxation.

INTRODUCTION

Some structurally related plant secondary metabolites such as the benzyltetrahydroisoquinolines norarmepavine, coclaurine and norcoclaurine occur in many plants and are commonly found in those plants which synthesize bisbenzyltetrahydroisoquinolines (BBIs). In the past few years it has been shown that this class of alkaloids possesses a broad array of pharmacological actions (Pachaly, 1990) and therapeutic applications, although this last aspect has been almost exclusively circumscribed to oriental traditional medicines.

BBI alkaloids constitute a series of almost 400 tyrosine-derived metabolites with a rich and varied chemistry and pharmacology (Schiff, 1991). These alkaloids, derived from the 1-benzyltetrahydroisoquinoline skeleton, are some of the most attractive secondary metabolites of flowering plants and they are mainly found in members of so-called 'primitive' Angiosperms such as *Rhamnaceae, Berberidaceae* and *Annonaceae,* among other families (Torres, 1988). From a pharmacological point of view, the best known BBIs are: tetrandrine, isotetrandrine (*Menispermaceae*), antioquine (*Annonaceae*), berbamine and 7-0-desmethylisothalicberine (*Berberidaceae*) and many publications have shown that they act as calcium antagonists (D'Ocon *et al.*, 1989, 1992; Fang and Jiang, 1986; Herman and Chadwick, 1974; Manwen *et al.*, 1982; Martínez *et al.*, 1997; Morales *et al.*, 1989, 1993).

Tetrandrine, a *bis*-coclaurine derivative, is the most active in most systems tested. It has antihypertensive, antianginal and antiarrhythmogenic action and has been advocated for a variety of medical purposes including its use as a diuretic, expectorant, cathartic, antiinflammatory and analgesic (Herman and Chadwick, 1974; Department of Pharmacology, Wuhan Medical College, 1979). More advanced studies, using the voltage-clamp and patch-

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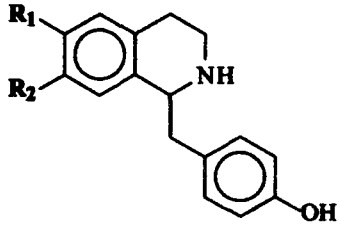
clamp techniques, have postulated that tetrandrine acts on L-and T-type calcium channels (King *et al.*, 1988; Liu *et al.*, 1991; Rubio *et al.*, 1993).

Tetrandrine has been isolated from many species of *Stephania* and *Cyclea,* and the evidence indicates that its biosynthetic pathway is: tyrosine → norcoclaurine → coclaurine → *N*-methylcoclaurine.

Tetrandrine is formed by oxidative dimerization of *N*-methylcoclaurine (Bhakuni *et al.*, 1980). It is already known that *N*-methylcoclaurine is contained in a diversity of plant families. Among them, it occurs in perhaps one of the most useful Chinese medicinal plants, *Stephania tetrandra,* used in China to treat angina and hypertension. The pharmacological information about *N*-methylcoclaurine suggests that this alkaloid is able to induce calcium antagonist effects on rat isolated atria and vas deferens. Unfortunately, the information is scanty and has been published almost exclusively in Chinese journals (Huang *et al.*, 1988), often being available only in the form of brief summaries from *Chemical Abstracts.*

Coclaurine is widely found in many plant species and presumably is the universal precursor of isoquinoline alkaloids. Among many other species, it occurs in *Peumus boldus* (Asencio *et al.*, 1993) a medicinal plant of Chile, currently used in infusions to treat digestive and hepatobiliary disorders, *Magnolia salicifolia* Maxim., well known for its cardiovascular effects and in *Annona cherimolia,* a medicinal plant used alone or mixed with *Psidium guajaba* L. (Morales and Lozoya, 1994; Morales *et al.*, 1994) in Mexico, Central America and other regions for its antidiarrhoeic effects (Aguilar *et al.*, 1994). Coclaurine exhibits antispasmodic activity on isolated uterus and induces negative inotropism on cardiac preparations. These effects have supported the proposition of coclaurine as a calcium antagonist (Kimura *et al.*, 1989; Martin *et al.*, 1993). In addition, it has recently been reported that coclaurine inhibited arachidonic acid-induced platelet aggregation (Chen *et al.*, 1996).

The chemical structure of coclaurine differs from that of norcoclaurine in that it has a methoxy group at the 6-position of the tetrahydroisoquinoline skeleton, while norcoclaurine has a hydroxyl group at this position (see Fig. 1).



	R_1	R_2
Norcoclaurine	OH	OH
Coclaurine	CH_3O	OH
Norarmepavine	CH_3O	CH_3O

Figure 1. Chemical structures of the benzylisoquinolines norcoclaurine, coclaurine and norarmepavine. These structures differ by the presence of two chemical groups at positions noted as R_1 and R_2 on the schematic representation of the tetrahydroisoquinoline skeleton.

Norcoclaurine (higenamine or demethylcoclaurine) postulated as the central intermediate in benzylisoquinolines alkaloid biosynthesis (Stadler *et al.*, 1989), has been found in *Annona squamosa* used in the traditional medicine of Africa, India and the Far East (Cavé, 1986; Evans, 1991), and in *Aconitum carmichaeli,* used to prepare the traditional Sino-Japanese medicine *Bushi* (Xiao, 1991). It has been reported elsewhere that this traditional medicine induces alterations such as bradycardia, irregular cardiac rhythm and cardiac arrest. Furthermore, a case has been reported in which resuscitation was unsuccessful and a man aged 30 years died 15 hours after *Bushi* ingestion (Fatovich, 1992). In studies carried out with guinea-pig isolated papillary muscle, norcoclaurine was postulated as a calcium agonist. Besides showing a positive inotropic effect, the alkaloid induced a parallel shift to the left of the Ca^{2+} curve and it had a tendency to shift to the left the isoproterenol-induced response curve (Kimura *et al.*, 1989). The authors suggested that norcoclaurine was inhibited by coclaurine in an apparently competitive manner, meaning that both alkaloids were acting in the same site. In a series of recent publications, Kimura *et al.* (1994, 1996) have postulated norcoclaurine is a β_1-adrenoceptor agonist. This property could explain its stimulating effect on the heart. However, it still remains to be resolved how norcoclaurine could also be a calcium agonist when it induces aortic relaxation, a question enunciated elsewhere by other authors (Chang *et al.*, 1994).

Norarmepavine, a benzylisoquinoline which carries two methoxy groups at the 6-and 7-positions (Fig. 1), has received less attention. It has been found in many species belonging to *Berberidaceae, Papaveraceae, Celastraceae* and *Rhamnaceae* (Torres, 1988). A few years ago it was reported that norarmepavine competitively antagonizes the uterine muscular contractions induced by acetylcholine and calcium (Martin *et al.*, 1993). In other works it was demonstrated that norarmepavine and coclaurine possess poor antioxidative properties (Cassels *et al.*, 1995) and very weak trypanocidal effects (Morello *et al.*, 1994).

In the present work, novel effects of norarmepavine on rat cardiovascular parameters and on rat isolated atria are presented. Furthermore, we decided to compare the effects of norarmepavine with those of coclaurine and norcoclaurine on aortic smooth muscle, a model useful for studying calcium modulators (Morales *et al.*, 1995), to contribute to the knowledge of the pharmacological effects of these structurally similar alkaloids.

MATERIAL AND METHODS

***In vivo* experiments.** Young adult Sprague-Dawley rats of both sexes, weighing 200–350 g were used. Food and water were given *ad libitum.* Rats were anaesthetized with nembutal, 50 mg/kg *i. p.*, the trachea was exposed by a mid-line incision and a respiratory cannula was inserted. The femoral artery and vein were cannulated and catheterized with PE90 and PE50 Clay Adams polyethylene tubing, respectively. After 15 min of stabilization each rat was injected with norarmepavine for 1 min and the injection volume was kept constant (1 mL/kg). Norarmepavine was administered in three

doses (5, 7.5 and 10 mg/kg *i. v.*) dissolved in 0.9% NaCl. The drug was infused (0.1–0.5 mL/min) through the vein. The femoral artery was connected to a Nihon Khoden polygraph, through a Gould Model P23 IDS Statham pressure transducer to record arterial blood pressure. The duration of the hypotensive effect was taken as the time elapsed from drug injection until the mean arterial pressure (MAP) value became indistinguishable from the control. Heart rate (HR) was calculated from DII or DIII EKG derivatives. The animals were maintained on a thermoregulated bed throughout the experimental procedure. Rectal temperature was continuously monitored with a Simpson Electric Model 43 telethermometer. The MAP and HR were measured 3 min after drug injection and compared with basal values.

Chronotropy in rat isolated atria. After cerebral contusion, the heart of each rat ($n = 7$) was removed and the isolated atria were deposited in a 10 mL bath filled with Krebs solution at 32°C, continuously bubbled with 95% O_2, 5% CO_2. Krebs solution was (mM): NaCl, 117; $CaCl_2$, 1.84; $MgCl_2$, 0.55; KCl, 5.9; $NaHCO_3$, 25.0; Na_2HPO_4, 0.96; glucose, 11.1. The pH was adjusted to 7.4. One of the ends of the atrial strip was connected to the bottom of the bath and the other to a Grass FT03 force-displacement transducer. The strips were allowed to equilibrate for 30 min under a basal tension of 2 g, and then the frequency of beating was determined. This was accomplished by displaying the transducer signal on a oscilloscope (Nihon Khoden AP620-G), adjusting the scanning velocity to 2.5 div/s. The concentration-response curve was constructed through the cumulative addition of norarmepavine (10^{-7}–10^{-3} M) to the solution bathing the tissue. IC_{50} (concentration producing 50% inhibition of peak spontaneous beating frequency) was estimated by plotting the results according to the procedures described elsewhere (Fleming *et al.*, 1972).

Aortic relaxation experiments. The experimental models used in the present study are based on a methodology described elsewhere (Morales and Lozoya, 1994; Morales *et al.*, 1994), or otherwise indicated. Sprague-Dawley rats of both sexes, weighing 200–350 g were used. A section of the thoracic aorta was carefully cleaned of fat and connective tissue. Three aortic rings (3–5 mm) were excised and the endothelial lining was mechanically removed; afterwards, the rings were mounted on stainless-steel hooks in glass chambers for isolated tissues and attached to a Grass FT03 force-displacement transducer to record its isometric contraction on a Grass 7D polygraph. The aortic rings were stabilized in the tissue bath for 40 min under an optimal resting tension of 1.5 g. The baths were filled with 10 mL of a modified Krebs–Henseleit solution (KHS) with the following composition (mM): NaCl 122.0; KCl 4.7; $CaCl_2$ 2.0; $MgCl_2$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 15.0; glucose 11.5 and EDTA 0.026. The solution was maintained at 37°C and bubbled continuously with 95% O_2, 5% CO_2 at pH 7.4. The preparations were contracted three times with a depolarizing high K^+ solution of the following composition (mM): NaCl 56.7; KCl 70.0; $CaCl_2$ 2.0; $MgCl_2$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 15.5; glucose 11.5 and EDTA 0.026 at pH 7.4. When the last K^+-induced contraction reached a steady maximal response, cumulative concentration *versus* aortic-relaxation curves for coclaurine, norcoclaurine, and norarmepavine (10^{-7} to 10^{-3} M) were obtained. Relaxation was expressed as a percentage decrement of the maximum tension obtained by K^+ depolarization.

Aortic contraction experiments. After a third KCl-induced contraction, the aorta was relaxed with KHS. Ten minutes after the relaxation, the bath solution was replaced with a high K^+, Ca^{2+}-free KHS. To preserve the isotonicity of the solution, calcium was substituted by sodium. Cumulative concentration *versus* contraction curves (control) were obtained by stepwise increases in Ca^{2+} (10^{-5}–10^{-2} M). Afterwards, the same arterial segments were previously treated with (±)-coclaurine, (±)-norcoclaurine and (±)-norarmepavine before the addition of calcium. Tension developed was expressed as a percentage of the maximum tension obtained by K^+ depolarization in each aortic ring. The temperature of the bath was maintained at 37°C.

In every concentration-response curve the concentration producing 50% of maximal relaxation (RC_{50}) or 50% of maximal contraction (EC_{50}) was determined according to methods described elsewhere (Fleming *et al.*, 1972). Removal of the endothelium was functionally verified at the end of each experiment by recording 10^{-4} M acetylcholine-induced relaxation on the rings previously contracted by 10^{-6} M norepinephrine (Furchgott and Zawadzky, 1980). When the endothelial lining was effectively removed, arterial segments did not relax under the influence of acetylcholine.

Statistics. The statistical analysis of the results was performed using the mean values ± standard error of the mean (SEM). Significance was determined by Student's *t*-test for paired data and it was accepted at $p < 0.05$.

Synthesis. (±)-Coclaurine (1-(*p*-hydroxybenzyl)-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline) was synthesized according to Teitel and Brossi (1968), and (±)-norarmepavine (1-(*p*-hydroxybenzyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline) was prepared by a similar procedure, using homoveratrylamine instead of homovanillylamine. Both racemic alkaloids were used as the hydrochlorides. (±)-Norcoclaurine hydrobromide (1-(*p*-hidroxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) was prepared by refluxing coclaurine in 48% hydrobromic acid and removing excess acid and water. The salts were recrystallized to homogeneity, and their purity was checked in all cases by TLC and by high resolution ^1H NMR.

Indicated concentrations are those which were obtained after dilution of stock aliquots in the organ bath containing 10 mL of Krebs solution.

RESULTS AND DISCUSSION

Cardiovascular effects of (±)-norarmepavine

Under control conditions, normotense anaesthetized rats exhibited a MAP and a HR of 108.6 ± 6.1 mmHg and 444.0 ± 14.7 beats/min, respectively. Both parameters showed a dose-dependent modification within 2–3 min of *i.v.* administration of (±)-norarmepavine (Fig. 2). At the lowest dose (5 mg/kg), norarmepavine decreased blood pressure by an average of 21 mmHg and HR remained

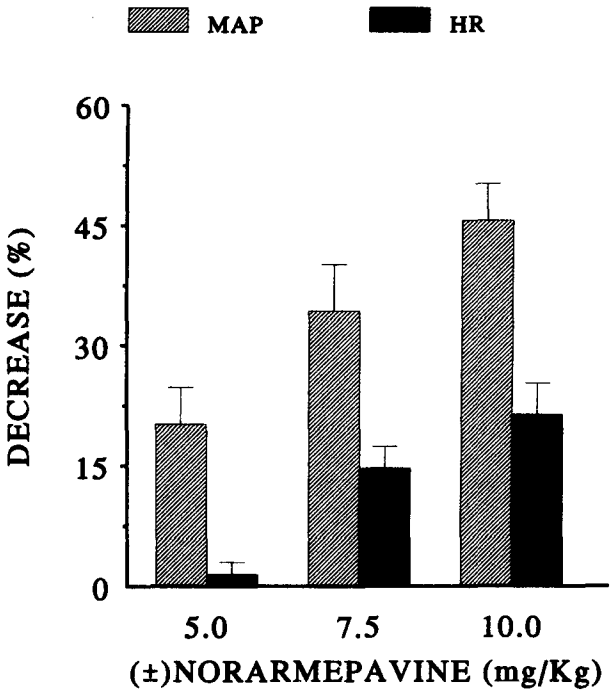


Figure 2. *In vivo* effects of norarmepavine on the mean arterial pressure (MAP) and heart rate (HR) in normotense anaesthetized rats. Data are expressed as a percentage of response decrease with respect to the mean control values ± SEM ($n = 7$).

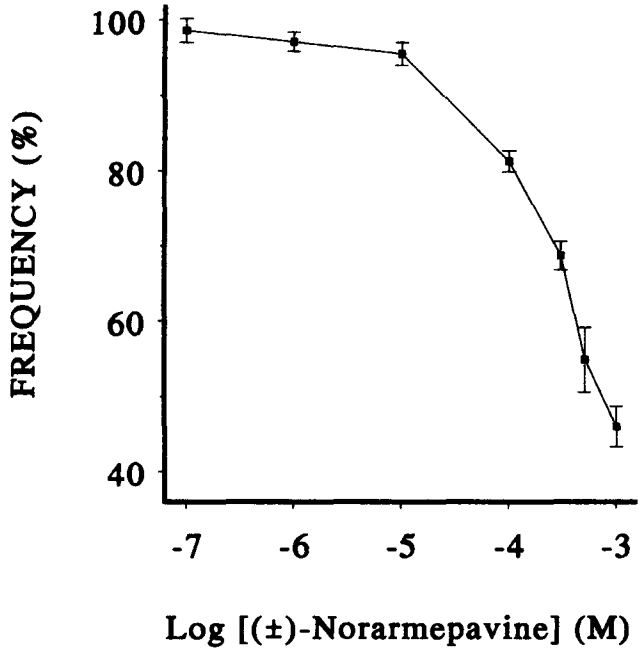


Figure 3. Norarmepavine cumulative effects on spontaneously beating rat-isolated atria. Control beating frequency was 232.6 ± 5.9. Data are expressed as a percentage of the control frequency. Each point represents the mean ± SEM ($n = 7$).

almost unaltered. With a dose of 10 mg/kg, MAP and HR decreased 48 mmHg and about 90 beats/min, respectively. The duration of the hypotensive effect was variable and never exceeded 8 min, even with the higher doses. Hypotensive episodes raised no reflex tachycardia. These results made evident the (±)-norarmepavine hypotensor and bradycardic effects *in vivo*.

Effects of (±)-norarmepavine on right isolated atrium

(±)-Norarmepavine induced a concentration-dependent negative chronotropic effect on spontaneously beating right atrium. The range of concentrations which induced frequency modification was rather narrow, beginning at 10^{-5} M (4.5%) and became 54% at 10^{-3} M, as depicted in Fig. 3. IC_{50} value was estimated as 1.5×10^{-4} M ($n = 7$). These results show that (±)-norarmepavine induced bradycardia *in vivo* could be exerted by a direct inhibitory action on atrial and/or excito-conductor tissues. In this sense, (±)-norarmepavine effects are similar to the reported effects of coclaurine on heart and absolutely different to the calcium channel activator or β_1-adrenergic agonist role proposed for (±)-norcoclaurine (Kimura *et al.*, 1989, 1994).

Effect of verapamil, norarmepavine, coclaurine and norcoclaurine on the KCl-induced contractions

As a control for assessing the external calcium dependence of the KCl-induced contraction of rat aortic smooth muscle, the calcium channel antagonist verapamil (10^{-10}–10^{-5} M) evoked a concentration-related relaxation and completely relaxed the aortic preparations at 10^{-5} M. Verapamil RC_{50} was determined as 1.2×10^{-8} M ($n = 6$), close to those values reported elsewhere (Morales *et al.*, 1995). Maximal KCl-induced aortic contraction was about 1.51 ± 0.08 g of tension per g of wet tissue ($n = 30$).

(±)-Norarmepavine, (±)-coclaurine and the calcium channel antagonist (±)-verapamil produced a 100% relaxation of the contractions induced by KCl. (±)-Norarmepavine- and (±)-coclaurine-induced aortic relaxations were concentration-dependent in the range 10^{-6} to 10^{-3} M. The concentration-response curves are shown in Fig. 4. The RC_{50} for (±)-norarmepavine and (±)-coclaurine were 4.4×10^{-5} ($n = 5$) and 8.2×10^{-5} M ($n = 9$), respectively.

(±)-Norcoclaurine relaxation of the aortic rings started from a lower concentration (10^{-7} M, 4.2%), however, the maximal relaxation attained was 46.7% ± 7.4% ($n = 6$), at 10^{-3} M. The RC_{50} for (±)-norcoclaurine was estimated as 7.5×10^{-5} M.

These results demonstrate that (±)-norarmepavine, (±)-coclaurine and (±)-norcoclaurine have relaxant activity and corroborate those of previous studies on other benzylisoquinoline alkaloids (Ivorra *et al.*, 1992) in that an increase in the degree of methylation of the hydroxy groups enhances the relaxant activity.

In addition, the rank order of potency was: (±)-verapamil > (±)-norarmepavine > (±)-norcoclaurine > (±)-coclaurine.

Comparatively, (±)-norarmepavine potency was very similar to (±)-glaucine and slightly greater than (S)-boldine and (R)-apomorphine, benzylisoquinolines with calcium and α_1-adrenergic antagonistic properties (Ivorra *et al.*, 1992, 1993).

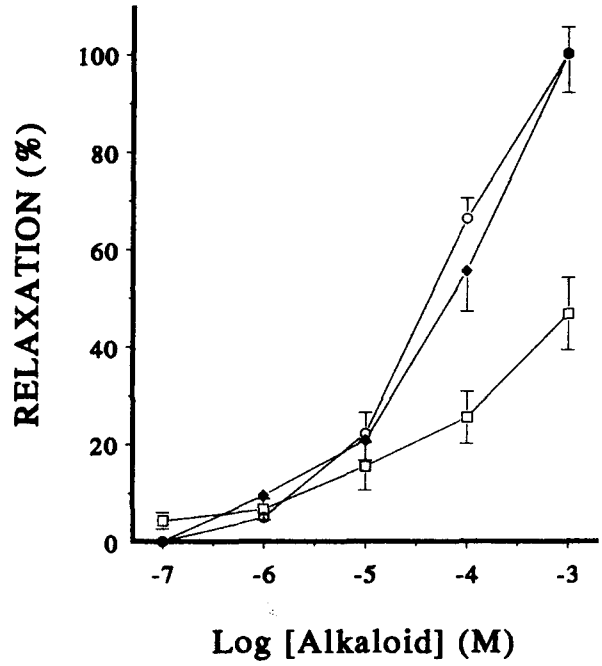


Figure 4. Alkaloid concentration-response curves. Both coclaurine (◆, $n = 9$), norcoclaurine (□, $n = 6$), and norarmepavine (○, $n = 5$) show relaxant activity on rat aortic rings previously contracted with 70 mM KCl. Each point represents the mean ± SEM.

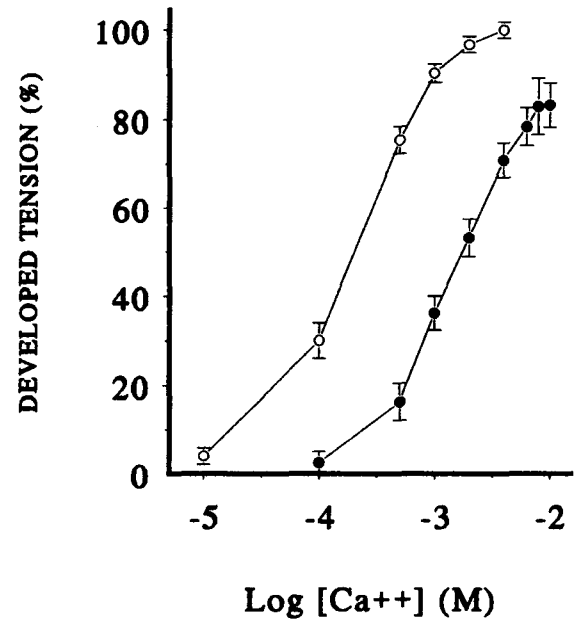


Figure 5. Calcium concentration-contraction curve on rat aortic rings with (●) and without (○) 80 μ M norarmepavine. Data represent the mean ± SEM ($n = 5$).

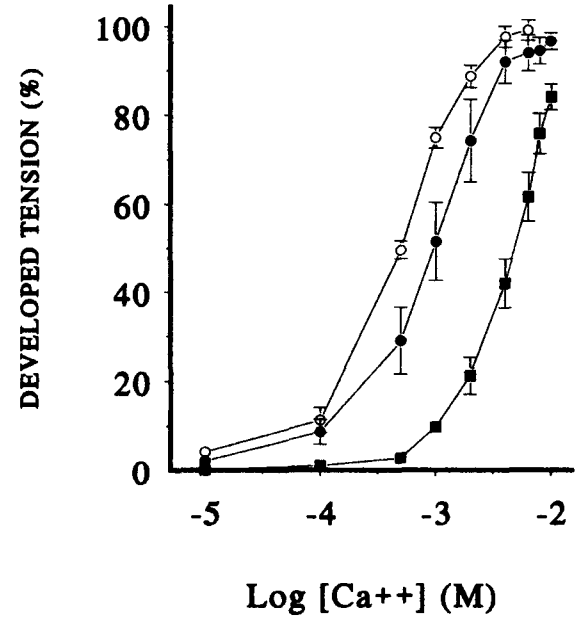


Figure 6. Calcium concentration-contraction curve on rat aortic rings with and without norcoclaurine. Data values of control (○, $n = 10$), 0.1 mM norcoclaurine (●, $n = 4$), and 1 mM norcoclaurine (■, $n = 4$) represent the mean ± SEM.

Benzylisoquinolines effects on calcium-contraction curves

(±)-Norarmepavine, (±)-coclaurine and (±)-norcoclaurine shifted to the right the control concentration-contraction curve for $CaCl_2$ acting as calcium antagonists. Calcium concentration inducing half maximal contraction (Ca^{2+} EC_{50}) increased about 12-fold by the addition of either 8×10^{-5} M (±)-norarmepavine (Fig. 5) or 8×10^{-5} M (±)-coclaurine (not shown). There was no statistically significant difference between the effect of both alkaloids.

On the other hand, (±)-norcoclaurine exhibited a lower antagonistic potency. In the presence of 1×10^{-4} and 1×10^{-3} M (±)-norcoclaurine, Ca^{2+} EC_{50} increased about 2-fold and 14-fold respectively, according to the curves shown in Fig. 6. The calcium antagonist-like effect of (±)-norcoclaurine was about 1 order of magnitude less potent than that of (±)-norarmepavine and (±)-coclaurine.

From the results obtained in the present study we can conclude that the *in vivo* hypotensive effect of (±)-norarmepavine may arise as a consequence of its capability to induce relaxation of peripheral vascular smooth muscle and its simultaneous bradycardic effects could be explained by a direct negative chronotropic action on atria. This last effect could also be masking the characteristic tachycardia elicited by sympathetic reflex.

It is proposed that this alkaloid is modulating either calcium entry, its intracellular release or the calcium sensitivity of the cell contractile-machinery, not excluding other possible mechanisms at an intracellular level. In this sense, (±)-norarmepavine cardiovascular effects resemble more closely (±)-coclaurine pharmacological effects than (±)-norcoclaurine, and it is correlated with a greater similarity between the chemical structures of these two benzyltetrahydroisoquinolines.

It is still open to debate whether (±)-norcoclaurine, having OH instead of CH_3O groups, could exert its cardiovascular actions by more than one mechanism. In this sense, we agree with those authors who have proposed a calcium antagonist action to explain (±)-norcoclaurine-induced vascular relaxation (Chang *et al.*, 1994). In contrast, (±)-norcoclaurine cardiac effects have been mostly attributed to calcium channel activation and β_1-adrenoceptor agonism (Kimura *et al.*, 1989, 1994,

1996). From the results obtained in vascular preparations, the proposal that (±)-norcoclaurine is a calcium channel activator is untenable. Instead, we consider that a probable phosphodiesterase type IV inhibition, as has been proposed for other benzylisoquinolines (Ivorra *et al.*, 1993), deserves to be studied in depth. It could be a better possibility to explain (±)-norcoclaurine-induced cardiac and vascular effects through a common mechanism.

Another explanation is that both (+) and (−)-coclaurine isomers could act through opposing mechanisms as has recently been demonstrated for R-(+)- and S-(−)-hyosciamine, a well known *Hyoscyamus niger*-derived cholinergic modulator (Ghelardini *et al.*, 1996). There exists the possibility that until now no success has been obtained in resolving the racemic composition of norcoclaurine used in pharmacological experiments.

In another aspect, the identification of endogenous codeine and thebaine in animal tissues has recently been reported (Hosztafi and Fürst, 1995) and it has been suggested that S-norcoclaurine could be an opioid precursor in the brain of rats with chronical ethanol ingestion (Haber *et al.*, 1997). This finding warrants further research on this alkaloid which seems to be a metabolite common to plants and mammals.

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