Mechanism of the cardiovascular activity of laudanosine: comparison with papaverine and other benzyloisoquinolines

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1 The activity of ($\pm$)-laudanosine, a benzyltetrahydroisoquinoline alkaloid, was investigated in pithed rats and rat isolated aorta. Its effects on [3 H]-prazosin, [3 H]-(+)-*cis*-diltiazem and [3 H]-nitrendipine binding to rat cerebral cortical membranes, and on the different molecular forms of cyclic nucleotide phosphodiesterases (PDE) isolated from bovine aorta were investigated.

2 The dose-response curve to methoxamine (3–300 $\mu\text{g kg}^{-1}$, i.v.) in normotensive pithed rats was shifted to the right by ($\pm$)-laudanosine, 3 and 6 mg kg^{-1} .

3 ($\pm$)-Laudanosine inhibited in a concentration-dependent manner the contractile responses evoked by noradrenaline (NA 1 μM), depolarizing solution (KCl 80 mM) or depolarizing solution plus phentolamine (10 μM) in rat isolated aorta. The alkaloid appeared to be more potent against NA-induced contractions.

4 In Ca^{2+} -free solution, ($\pm$)-laudanosine (100 μM) inhibited the contraction evoked by NA and did not modify the phasic contractile response evoked by caffeine. The alkaloid did not modify the refilling of the intracellular Ca^{2+} -stores sensitive to NA or caffeine.

5 ($\pm$)-Laudanosine inhibited [3 H]-prazosin binding to cortical membranes and also inhibited [3 H]-(+)-*cis*-diltiazem but with a lower potency. [3 H]-nitrendipine binding was not affected by laudanosine.

6 ($\pm$)-Laudanosine does not have a significant effect on the different forms of PDEs isolated from bovine aorta. In contrast, compounds structurally related to this alkaloid such as papaverine and its derivatives, had a non-selective or more specific inhibitory effect on these PDE forms. These differences can be explained on the basis of their structural features: the planarity of the isoquinoline ring (papaverine) facilitates the interaction with receptor sites, and the different position of the benzyl group does not modify the activity unless this position leads to the presence of a chiral centre (laudanosine).

7 These results suggest that ($\pm$)-laudanosine has a selective activity as an α_1 -adrenoceptor blocker. Its lack of action on different PDE forms provides us with information about a group of benzyloisoquinolines that with small structural changes show a different effect on PDE-forms isolated from vascular smooth muscle.

Keywords: Pithed rat; rat aorta; calcium antagonists; α_1 -adrenoceptors blocking agents; benzyltetrahydroisoquinolines; phosphodiesterase inhibitors

Introduction

Recently, new structural classes of compounds related to the benzyltetrahydroisoquinoline structure have been reported to be Ca^{2+} channel blockers (King *et al.*, 1988; Triggle *et al.*, 1989; D'Ocon *et al.*, 1989; 1991; Ming Lu & Quing Lu, 1990; Lacroix *et al.*, 1991). Some of them, such as tetrandrine, laudanosine, glaucine and cularines, show a mechanism of uterine smooth muscle relaxant activity which is different from papaverine in spite of their structural relationship (D'Ocon *et al.*, 1991; Anselmi *et al.*, 1992). Papaverine acts at the intracellular level by a non-specific mechanism, whereas benzyltetrahydroisoquinolines relax smooth muscle by a more specific mechanism, including Ca^{2+} entry blocking activity, and have no effect on contractile machinery, Ca^{2+} -release or Ca^{2+} -redistribution from intracellular storage sites. This different interaction might be attributed to the geometry as well as to differences in the basicity of the heteroatom and could be responsible for the different behaviour of these compounds.

The aim of the present work was to investigate the mechanism of the relaxant action of a benzyltetrahydroisoquinoline, laudanosine, in vascular smooth muscle and compare it to the relaxant action of papaverine.

Our previous studies with rat aorta showed that some aporphines with a benzyltetrahydroisoquinoline structure such as glaucine, boldine and apomorphine, possess not only Ca^{2+} entry blocking properties but also α_1 -adrenoceptor antagonist activity, and therefore inhibit [3 H]-(+)-*cis*-diltiazem and [3 H]-prazosin binding, relax rat aorta previously contracted by addition of noradrenaline or KCl and inhibit some forms of cyclic AMP-phosphodiesterases (Ivorra *et al.*, 1992a,c). The present work includes *in vivo* experiments in pithed rats and functional studies on rat isolated aorta contracted by addition of noradrenaline, caffeine or KCl. In order to assess whether laudanosine interacts directly with Ca^{2+} channel receptors or α -adrenoceptors, we examined the effects of the alkaloid on [3 H]-nitrendipine, [3 H]-(+)-*cis*-diltiazem and [3 H]-prazosin binding to rat cerebral cortical membranes.

The present study also examines the question of whether laudanosine has any effects on cyclic nucleotide phosphodiesterase (PDE) activity, as papaverine does. In mammalian tissue cells, several distinct molecular forms of PDE exist (for

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review see Beavo, 1988). Accordingly, we examined the ability of laudanosine to inhibit separate PDE enzymes isolated from bovine smooth muscle, and compared the results obtained to the action of a series of isoquinolines, structurally related to laudanosine, on PDE activity.

Methods

Pithed rat preparation

Rats (300–340 g) received atropine (1 mg kg⁻¹, i.p.) to prevent muscarinic activation; 15 min later they were pithed under diethyl ether anaesthesia by inserting a blunt steel rod (diameter 1.5 mm) through the orbit and foramen magnum down into the spinal canal (Shipley & Tilden, 1947). The trachea was cannulated and the animals were ventilated with room air (60 strokes min⁻¹, 20 ml kg⁻¹ body wt.) the left jugular vein was cannulated for drug administration and arterial blood pressure was measured by a cannula placed in the right carotid artery. Bilateral vagotomy was also performed. The rats were allowed to stabilize for 15 min before the experiment was begun. Methoxamine (3–300 µg kg⁻¹), a selective α₁-adrenoceptor agonist (Kobinger & Pichler, 1980) was injected in single doses and sufficient time was allowed between them for blood pressure to return to preinjection levels. Dose-response curves induced by methoxamine before and 7 min after drug infusion (2 min) were determined.

Values are expressed as means ± s.e.mean. The statistical significance between groups was assessed by ANOVA followed by a Student's *t* test. A value of *P* < 0.05 was taken as significant.

Functional study

Helically cut strips from the thoracic aorta of male Wistar rats (200–220 g) were prepared and mounted as described by Furchgott & Zawadzki (1980). Each preparation was suspended in a 10 ml organ bath containing Krebs-bicarbonate solution (KBS), maintained at 37°C and gassed with 95% O₂ and 5% CO₂. An initial load of 1 g was applied to each preparation and maintained throughout a 75–90 min equilibration period before agonist addition. Tension was recorded isometrically on a polygraph (Grass M7) via force-displacement transducers (Grass FT03).

KBS had the following composition (mM): NaCl 118, KCl 4.75, CaCl₂ 1.8, MgCl₂ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25 and glucose 11. Ca-free solution had the same composition except that CaCl₂ was omitted and EDTA (0.1 mM) was added.

The absence of relaxant response after 100 µM acetylcholine addition in preparations previously contracted with noradrenaline 1 µM indicated the absence of a functional endothelium (Furchgott & Zawadzki, 1980).

Concentration-relaxation response curves were obtained by the addition of cumulative concentrations of laudanosine (0.1 µM–100 µM) once the sustained contractions had been reached with KCl (80 mM) in the presence or absence of phentolamine (10 µM) and noradrenaline (1 µM). Relaxations were expressed as a percentage of the maximum tension obtained by agonist addition. *E*_{max} values representing the maximal relaxation obtained after addition of the highest dose of the alkaloid were calculated. The concentration needed to produce 50% inhibition (IC₅₀) was also calculated for each preparation from a least squares regression analysis of all points between 20–80% of the maximal response.

Figure 1a shows the experimental procedure used to study the action of laudanosine on noradrenaline-induced contraction in the absence of extracellular calcium at 37°C. In order to clarify the intracellular action of laudanosine similar experimental procedures were performed but NA was replaced by caffeine (Figure 1b). In Ca-free medium, caffeine yielded a contraction only at 25°C but not at 37°C, so in protocols

using caffeine as agonist, the temperature was decreased to 25°C.

After incubation for 1 h in KBS to stabilize the preparation, 1 µM noradrenaline or 10 mM caffeine was administered to contract the muscle, and the amplitude of the resulting contraction was monitored as a reference. The solution was then replaced by Ca²⁺-free solution and left for 15 min before 1 µM noradrenaline or 10 mM caffeine was added and the contractile response monitored as a measure of the amount of Ca²⁺ released from the store sensitive to the agonist. After washing, a new addition of the agonist, noradrenaline or caffeine, did not elicit any contractile response. The solution was then replaced by normal KBS and left for 20 min to refill the depleted Ca²⁺ stores. Noradrenaline or caffeine were again administered after 15 min in Ca²⁺-free solution and a new response to agonist was obtained similar in magnitude to the first one in Ca²⁺-free medium. The effect of alkaloids was tested on the last agonist-induced contraction (Figure 1) or on the refilling process (Figure 2).

Contractile responses in Ca-free KBS were expressed as a percentage of the noradrenaline- or caffeine-induced contraction in normal KBS.

Results are expressed as the mean ± s.e.mean of *n* preparations obtained from different animals. Statistical significance of differences between the means was assessed by Student's *t* test. *P*-values of less than 0.05 were considered to represent significant differences.

Radioligand binding experiments

Membranes were prepared from cerebral cortex of male Wistar rats as previously described (Schott *et al.*, 1988). Briefly, the tissue was homogenized in 10 vol (w/v) of ice-cold buffer (50 mM Tris-HCl, pH 7.5) in an ultra-turrax (twice, 15 s). The homogenate was centrifuged at 40,000 *g* at 4°C for 10 min and the resulting pellet was washed by resuspension and centrifugation under the same conditions. The final pellet

Figure 1 Experimental procedure used to study the effect of laudanosine or papaverine in the absence of extracellular calcium on noradrenaline-induced (NA) contraction (a) or caffeine-induced (Caff) contraction (b) in the absence of extracellular Ca²⁺. NA₁ or Caff₁: addition of the agonist after 15 min incubation in Ca²⁺-free medium. NA₁' or Caff₁': second addition of the agonist after washing (W) in Ca²⁺-free medium. NA₂ or Caff₂: addition of the agonist in presence or absence of the alkaloid 3 × 10⁻³ M, after 20 min resting period in KBS (Ca²⁺ 1.8 mM) and 15 min in Ca²⁺-free solution. NA₃: addition of the agonist after a resting period in KBS (20 min).

Figure 2 Experimental procedure used to study the effect of laudanosine or papaverine on the refilling of intracellular calcium stores previously depleted by noradrenaline (NA) (a) or caffeine (Caff) (b). NA₁ or Caff₁: addition of the agonist after 15 min incubation in Ca²⁺-free medium. NA₁' or Caff₁': second addition of the agonist after washing (W) in Ca²⁺-free medium. NA₂ or Caff₂: addition of the agonist after 20 min resting period in KBS (Ca²⁺ 1.8 mM) in presence or absence of the alkaloid 3×10^{-5} M, and 15 min in Ca²⁺-free solution.

was resuspended in incubation buffer to give a final protein concentration of about 1 mg ml⁻¹. Membrane aliquots (250 µl) were incubated in a final volume of 500 µl in 50 mM Tris-HCl (pH 7.5) with [³H]-prazosin (0.1–0.2 nM) or [³H]-nitrendipine (0.3–0.4 nM) or in 50 mM Tris-HCl pH 7.5 containing bovine serum albumin (BSA) 1 mg ml⁻¹ with [³H]-(+)-*cis*-diltiazem (3–4 nM), in the absence or in the presence of drugs at various concentrations.

Incubations were carried out at 25°C for 30 min ([³H]-prazosin), 90 min ([³H]-nitrendipine) or 120 min ([³H]-(+)-*cis*-diltiazem), after which the reaction was terminated by the addition of 3.0 ml ice-cold buffer and rapid filtration over Whatman GF/B filters that were washed with 9 ml (3 × 3 ml) of ice-cold buffer. For [³H]-(+)-*cis*-diltiazem binding experiments, filters were pretreated for 120 min with polyethylenimine 0.3%. The radioactivity bound to the filters was determined by liquid scintillation counting. Non-specific binding was determined in the presence of phentolamine (10⁻⁶ M) nifedipine (10⁻⁶ M) or diltiazem (10⁻⁵ M), respectively. All determinations were performed in triplicate.

Displacement curves were analysed by a computerized non-linear curve-fitting program (Graph-Pad) and K_i values were calculated from the formula of Cheng & Prusoff (1973).

Phosphodiesterase inhibition

Cytosolic cyclic nucleotide phosphodiesterases (PDE) were isolated from the media layer of bovine aorta by a modification of the methods of Lugnier *et al.* (1986). Briefly, the tissue was homogenized in 10 vol (w/v) isotonic buffer A (25 mM sodium phosphate pH 6.6; 250 mM saccharose; 2 mM magnesium acetate (MgAc); 1 mM dithiothreitol; 5 mM ethylene glycol bis (β-aminoethylether) N,N,N',N' tetraacetic acid (EGTA); 2000 u ml⁻¹ aprotinin; 10 µg ml⁻¹ soya bean trypsin inhibitor and 10 µg ml⁻¹ leupeptin) using an ultraturax (six times, 10 s) and a glass pestle homogenizer, and then centrifuged at 105,000 g for 60 min. The resulting super-

natant fraction was applied to a diethylaminoethyl (DEAE)-Sephacel ion-exchange column and eluted by buffer B (25 mM phosphate pH 6.6; MgAc 2 mM and dithiothreitol 1 mM) until no absorbance was detected in the eluate at 280 nm. Elution was then continued with a linear gradient of 0–0.55 M of NaCl in buffer B (flow rate 25 ml h⁻¹). Each fraction was tested for PDE activity.

Since DEAE-Sephacel did not allow the separation of calmodulin-PDE (type I) from cyclic GMP-PDE (type V) as did DEAE-Trisacryl chromatography (Lugnier *et al.*, 1986), a further high performance liquid chromatography (h.p.l.c.) repurification was done. The first peak was then injected into an h.p.l.c. column (TSK-DEAE-5PW), washed for 20 min with elution buffer (25 mM phosphate pH 6.6) and eluted (0.8 ml min⁻¹) by a linear NaCl gradient (0.05–0.3 M) in elution buffer. Fractions under each PDE activity peak were pooled, dialyzed against buffer (20 mM Tris-HCl, 2 mM MgAc pH 7.5) and stored in aliquots at –80°C with bovine serum albumin (BSA).

PDE activities were measured by the two-stage assay described by Wells *et al.* (1975) at a substrate ([³H]-cyclic AMP or [³H]-cyclic GMP) concentration of 1 µM, in the following buffer: 50 mM Tris-HCl pH 7.5, 2 mM MgAc, 1 mg ml⁻¹ BSA and in the presence of 10 µM CaCl₂ and 18 nM calmodulin or in the absence of Ca²⁺ and calmodulin but with 1 mM EGTA. To prevent the influence of cross-contamination between CGI-PDE (Type III) and cyclic AMP-PDE (Type IV), the studies performed with these forms were always carried out in the presence of 100 µM rolipram or 100 µM cyclic GMP, respectively.

The IC₅₀ (concentration which produced 50% inhibition of substrate hydrolysis) for the compounds studied was determined from the concentration-response curves obtained with five concentrations of inhibitor and calculated by a non-linear regression. The apparent K_i values were obtained according to Cheng & Prusoff (1973). Results are expressed as mean ± s.e. mean of three determinations made in duplicate using three different enzymatic preparations.

Drugs and solutions

The following drugs were used: atropine sulphate, (–)-noradrenaline L-tartrate and acetylcholine (Merck); anhydrous caffeine, laudanosine, papaverine, cyclic AMP and cyclic GMP (Sigma Chemical Co); phentolamine methanesulphonate (Ciba-Geigy), methoxamine HCl (Gayoso Wellcome), diethyl ether (Panreac).

[³H]-prazosin (20.2 Ci mmol⁻¹), [³H]-nitrendipine (70 Ci mmol⁻¹), [³H]-cyclic AMP (30–50 Ci mmol⁻¹) and [³H]-cyclic GMP (5–15 Ci mmol⁻¹) were purchased from the New England Nuclear. [³H]-(+)-*cis*-diltiazem (154 Ci mmol⁻¹) was obtained from Amersham.

Other reagents were of analytical grade.

Caffeine was dissolved in Ca-free KBS. The other drugs were dissolved in saline adjusted to pH 7.

Results

Pithed rats

The resting diastolic blood pressure of normotensive pithed rats was 47 ± 3.9 mmHg, *n* = 15. Intravenous administration of methoxamine (3 to 300 µg kg⁻¹) to control rats caused a dose-dependent increase in diastolic blood pressure (DBP) (Figure 3a and b). Injection of papaverine but not laudanosine at 3 and 6 mg kg⁻¹ induced a transient decrease of blood pressure (10–15 mmHg) with a return to basal levels in 2–3 min. The dose-response curve induced by methoxamine was shifted to the right (Figure 3a) by 3 and 6 mg kg⁻¹ laudanosine. Lower doses (0.3–1 mg kg⁻¹) were without effect on methoxamine-induced DBP responses. Systemic administration of papaverine (3–6 mg kg⁻¹) diminished in a

dose-dependent manner the methoxamine ($100\text{--}300\text{ }\mu\text{g kg}^{-1}$)-induced DBP increases without affecting the pressure responses to lower doses of the α_1 -adrenoceptor agonist. However, the decrease by papaverine (3 mg kg^{-1}) of the pressor response to methoxamine ($100\text{ }\mu\text{g kg}^{-1}$) did not reach statistical significance. In addition, papaverine (0.3 and 1 mg kg^{-1}) did not modify the pressor responses to methoxamine (results not shown).

Figure 3 Increases in diastolic blood pressure in pithed rats induced by administration of single doses of methoxamine ($3\text{--}300\text{ }\mu\text{g kg}^{-1}$) before and 7 min after: (a) papaverine 3 mg kg^{-1} (●) or 6 mg kg^{-1} (□); (b) laudanosine 3 mg kg^{-1} (●) or 6 mg kg^{-1} (□). Papaverine has been evaluated on 4 animals and laudanosine on 6–11 animals. s.e.mean are shown by vertical bars. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Functional study

Effects of laudanosine and papaverine on noradrenaline- or KCl-induced contractions in KBS The contractile response of rat aorta elicited by depolarizing solution (KCl 80 mM) at 37°C was $792.9 \pm 34.3\text{ mg}$ ($n = 7$). The same contraction elicited in the presence of phentolamine (10^{-5} M) at 37°C was smaller ($590.8 \pm 43.1\text{ mg}$, $n = 7$, $74.3 \pm 3.8\%$ of the control).

Noradrenaline ($1\text{ }\mu\text{M}$) produced a sustained contraction in rat isolated thoracic aorta incubated in KBS at 37°C ($735.4 \pm 90.6\text{ mg}$, $n = 8$). This dose of noradrenaline has proved to be maximal for contraction in rat aorta (Noguera & D'Ocon, 1992).

Laudanosine ($0.1\text{--}300\text{ }\mu\text{M}$) produced concentration-dependent relaxations of KCl-depolarized rat aorta at 37°C both in the presence and absence of phentolamine, but the parameters (E_{max} and IC_{50}) of the concentration-response curves showed significant differences (Table 1) suggesting that the relaxant action of this alkaloid depends both on α -adrenoceptor blocking activity and a decrease in the Ca^{2+} entry through voltage-dependent channels.

Addition of cumulative concentrations of laudanosine ($0.1\text{--}100\text{ }\mu\text{M}$) to the aorta contracted by noradrenaline ($1\text{ }\mu\text{M}$) in KBS induced concentration-dependent relaxations confirming results obtained with the KCl-induced contraction but the IC_{50} was lower against NA-induced contraction (Table 1).

Papaverine caused relaxation of the KCl-, KCl plus phentolamine- or NA-induced contractile response of rat aorta and the E_{max} or IC_{50} were similar in each case (Table 1).

Effects of laudanosine and papaverine on noradrenaline- or caffeine-induced contractions in a Ca^{2+} -free medium After 15 min in Ca^{2+} -free solution, addition of noradrenaline ($1\text{ }\mu\text{M}$) induced a phasic contraction followed by a tonic one (NA_1). The addition of noradrenaline for a second time in Ca^{2+} -free solution (NA_2) induced a significantly lower contractile response in the presence of laudanosine and this response was totally abolished in the presence of papaverine ($100\text{ }\mu\text{M}$) (Table 2, Figure 1a).

At 25°C , caffeine 10 mM induced a rapid transient contraction in Ca^{2+} 1.8 mM solution ($210.1 \pm 21.6\text{ mg}$, $n = 16$). Addition of caffeine after 15 min of exposure to a Ca^{2+} -free solution yielded a phasic contraction (Caff_1). After washing, addition of caffeine did not induce any contractile response. After a loading period (20 min) in KBS to refill the intracellular Ca^{2+} -stores, caffeine-induced contraction in Ca^{2+} -free medium (15 min) was restored (Caff_2). Preincubation (10 min) with laudanosine ($100\text{ }\mu\text{M}$) did not modify the phasic contractile response induced by caffeine in Ca^{2+} -free medium whereas papaverine ($100\text{ }\mu\text{M}$) decreased this contraction (Caff_2 ; Table 2; Figure 1).

Effects of laudanosine and papaverine on the refilling of intracellular Ca^{2+} stores sensitive to noradrenaline or caffeine In order to clarify the intracellular action of laudanosine and

Table 1 Inhibitory potencies of alkaloids on contractions induced by noradrenaline $1\text{ }\mu\text{M}$ (NA), KCl (80 mM) or KCl (80 mM) plus phentolamine ($10\text{ }\mu\text{M}$) in rat aorta

		NA	KCl	KCl + pH	$\text{IC}_{50} (\text{KCl} + \text{Ph})/\text{IC}_{50} (\text{NA})$
Papaverine	$E_{\text{max}} (\%)$	111.5 ± 8.3 $n = 7$	104.8 ± 8.5 $n = 6$	100.1 ± 3.4 $n = 4$	
	$\text{IC}_{50} (\mu\text{M})$	3.7 ± 0.9	5.6 ± 0.9	3.2 ± 0.4	0.865
Laudanosine	$E_{\text{max}} (\%)$	94.4 ± 2.9 $n = 5$	$69.8 \pm 3.0^*$ $n = 6$	$56.5 \pm 2.9^*$ $n = 7$	
	$\text{IC}_{50} (\mu\text{M})$	11.5 ± 2.3	$37.2 \pm 5.1^*$	$80.6 \pm 8.0^*$	7.009

Values are means $\pm$ s.e.mean.

*Significant difference ($P < 0.001$) from the corresponding values on NA-induced contraction.

n = number of experiments.

Table 2 Effects of agents (10^{-4} M) on noradrenaline (NA) or caffeine (Caff) induced contraction of rat aorta in Ca^{2+} -free medium (experimental protocol see Figure 1)

Agonist		NA		Caff	
A ₁	phasic tonic	21.8 ± 2.3	23.2 ± 2.6	32.9 ± 4.9	44.0 ± 2.6
		10.3 ± 1.3	10.7 ± 2.5	—	—
A ₂ + alkaloid	phasic tonic	Laudanosine	Papaverine	Laudanosine	Papaverine
		7.9 ± 0.9*	0	38.1 ± 5.7	11.0 ± 1.1*
		5.6 ± 1.1*	0	—	—
		n = 7	n = 4	n = 5	n = 3

% are calculated from the initial contraction by agonist in Krebs solution.

Values are mean ± s.e.mean.

n = number of experiments.

* $P < 0.001$ from A₁.

Table 3 Effects of agents (10^{-4} M) on the refilling of intracellular Ca^{2+} -stores sensitive to noradrenaline (NA) or caffeine (Caff) (experimental protocol see Figure 2)

Agonist		NA		Caff	
A ₁	phasic tonic	24.3 ± 2.2	27.5 ± 3.6	47.7 ± 8.4	39.2 ± 1.5
		8.6 ± 1.2	11.4 ± 3.4	—	—
A ₂	Refilling phasic tonic	Laudanosine	Papaverine	Laudanosine	Papaverine
		21.3 ± 1.1	0	42.2 ± 9.8	—
		7.1 ± 1.1	0	—	—
		n = 6	n = 4	n = 6	n = 3

% are calculated from the initial contraction by agonist in Krebs solution.

Values are mean ± s.e.mean.

n = number of experiments.

Figure 4 Displacement curves of [^3H]-prazosin (O) or [^3H]-(+)-*cis*-diltiazem specific binding (●) by laudanosine. Each point is the mean from three experiments made in triplicate with s.e.mean shown by vertical bars.

papaverine, similar experimental procedures were carried out but the alkaloids were added during the loading period in Ca^{2+} -containing KBS (20 min) after depletion of the intracellular stores sensitive to noradrenaline or caffeine (Figure 2). In these experimental conditions, subsequent addition of noradrenaline or caffeine in Ca^{2+} -free solution (NA₂ or Caff₂) induced no contraction after papaverine treatment but similar contractile responses after loading in the presence of laudanosine (Table 3).

Binding assays

The interactions of laudanosine with [^3H]-prazosin and [^3H]-(+)-*cis*-diltiazem binding are shown in Figure 4. Laudanosine

fully inhibited [^3H]-prazosin binding to cortical membranes, with an inhibition constant of $5.92 \pm 0.69 \mu\text{M}$. The fact that the pseudo-Hill coefficient (slope factor) was not significantly different from unity ($n_H = 0.95 \pm 0.04$) suggests direct competition between laudanosine and the radioligand for a single common binding site. In addition, laudanosine inhibited binding of [^3H]-(+)-*cis*-diltiazem but with a lower potency ($K_i = 26.29 \pm 4.96 \mu\text{M}$) and with a similar pseudo-Hill slope ($n_H = 0.91 \pm 0.13$). This suggests that the interaction between laudanosine and [^3H]-(+)-*cis*-diltiazem is also a simple competition for a single common binding site.

[^3H]-nitrendipine binding was not affected by laudanosine in concentrations up to $100 \mu\text{M}$ (results not shown).

Inhibition of bovine aorta cyclic nucleotide phosphodiesterase enzymes

As shown in Table 4, laudanosine does not have a significant effect on the different forms of PDEs. In contrast, papaverine exerted a relatively non-selective inhibitory effect on all PDE forms although the inhibition of the calmodulin-sensitive form (PDE I) appeared weaker than that of the others.

Discussion

Papaverine, a benzyloquinoline alkaloid, is a well-known spasmolytic agent and its action is ascribed to inhibition of phosphodiesterases (Lugnier *et al.*, 1972; Bolton, 1979; Cumiskey & Feigenson, 1983), the subsequent increase in cyclic AMP and cyclic GMP levels and modification of intracellular Ca^{2+} distribution. Furthermore, there is evidence that it may also antagonize Ca^{2+} influx (Schneider *et al.*, 1975; Reinhardt *et al.*, 1977) and act on α -adrenoceptors (Ivorra *et al.*, 1992c).

The results of our previous work on the structure-activity relationship of benzyloquinoline alkaloids has made it possible to divide them into two groups, the first one containing those that have an unsaturated heterocyclic ring, like papaverine, and the second one formed by compounds with a

tetrahydroisoquinoline ring. The latter group exhibits a more specific activity (Cortes *et al.*, 1990; D'Ocon *et al.*, 1991; Anselmi *et al.*, 1992) and the selectivity implies that these tetrahydroisoquinoline alkaloids, unlike papaverine, act mainly on the Ca^{2+} influx from the extracellular medium without producing changes in the intracellular distribution of this ion. This more specific action on calcium entry, was accompanied by a loss in the activity of these alkaloids as PDEs inhibitors.

The above observations prompted us to examine the potential activity of laudanoline, an alkaloid with a benzyltetrahydroisoquinoline ring, and we assayed this alkaloid in the present study on different experimental models including *in vivo* and *in vitro* preparations.

Our *in vivo* experiments were carried out in the pithed rat which is devoid of central cardiovascular reflexes and has been proved to be a suitable model for studying the effects of drugs on α -adrenoceptors in the periphery. The results

Table 4 Inhibition constants (K_i μM) of agents on cyclic nucleotide phosphodiesterases isolated from bovine aorta

		CaM-PDE type I	cGMP-PDE type V	cAMP-PDE type IV
BIQ	Papaverine	27.5	1.08	0.89
	Compound 1	20	0.5	1.37
BDHIQ	Dihydropapaverine	>100	18.9	8.2
	Compound 2	>100	33.6	>100
BTHIQ	Laudanosine	>100	>100	>100
	Compound 3	>100	>100	95.0

Values are given as mean $\pm$ s.e. mean from three determinations obtained on different enzymatic preparations. Apparent K_i values were calculated from IC_{50} values according to Cheng & Prusoff (1973). Phosphodiesterase activity was assessed with $1 \mu\text{M}$ of [^3H]-cyclic GMP + $10 \mu\text{M}$ CaCl_2 + 18 nM calmodulin for CaM-PDE; [^3H]-cyclic GMP + 1 mM EGTA for cGMP-PDE and [^3H]-cyclic AMP + 1 mM EGTA for cAMP-PDE.
BIQ = benzyloisoquinoline; BDHIQ = benzyldehydroisoquinoline; BTHIQ = benzyltetrahydroisoquinoline.

obtained in this study with methoxamine, a selective α_1 -adrenoceptor agonist, suggest that laudanosine has α_1 -adrenoceptor antagonist activity in the pithed rat, results that agree with those obtained in rat isolated aorta (present study). However, when we studied the effect of papaverine on methoxamine-induced pressor responses the alkaloid behaved as a non-competitive antagonist.

We assayed the action of laudanosine on contractions of the rat aorta which are mediated by: (1) Ca^{2+} -entry through potential operated channels (KCl-induced contraction); (2) Ca^{2+} -entry induced by α -adrenoceptor activation (noradrenaline-induced contraction); (3) release of intracellular Ca^{2+} (EDTA-resistant response to noradrenaline or caffeine).

KCl has been shown to produce a biphasic contraction in the rat aorta (Sakata & Karaki, 1991) depending exclusively upon extracellular Ca^{2+} (Marriott, 1988). The fact that in the presence of phentolamine the contractile response induced by KCl is smaller also indicates participation of catecholamine release in the contraction elicited by depolarization in rat aorta.

Contractions induced by high concentrations of noradrenaline are also biphasic: the early fast component of contraction depends upon release of intracellular Ca^{2+} , while the slowly developing sustained phase of contraction depends upon influx of extracellular Ca^{2+} (Marriott, 1988). Accordingly, under Ca^{2+} -free conditions, a smaller contraction may be induced in the rat aorta by noradrenaline and this must be mediated by intracellular Ca^{2+} -release from internal stores (Noguera & D'Ocon, 1992, 1993). On repeated exposure to noradrenaline, this contraction was not observed, which suggests that the Ca^{2+} store was of limited size and that re-sequestration of released Ca^{2+} was relatively inefficient.

A similar contraction has been observed after addition of caffeine in Ca^{2+} -free medium, but different mechanisms are evoked. The noradrenaline-induced release of intracellular Ca^{2+} is attributable to α_1 -adrenoceptor activation (Chiu *et al.*, 1987; Daly *et al.*, 1990), whereas caffeine releases Ca^{2+} from the same intracellular store as noradrenaline does (Noguera & D'Ocon, 1992), but through a different mechanism (Itoh *et al.*, 1983; Sato *et al.*, 1988).

Laudanosine and papaverine relax the contractile responses of rat aorta to noradrenaline or KCl in KBS solution, but analysis of the E_{max} and $\text{IC}_{50}(\text{KCl})/\text{IC}_{50}(\text{NA})$ ratios provides information on selectivity (Table 1) and indicates that laudanosine exhibits greater inhibition of the contractile response induced by NA than KCl, whereas papaverine relaxes both agents in a non-selective way, as described in an earlier paper (Ivorra *et al.*, 1992c). The fact that the IC_{50} of laudanosine on the contractile response induced by KCl was different in the presence and absence of phentolamine indicates that the relaxant action shown by this alkaloid is complex and depends both on α -adrenoceptor blocking activity and a decrease in the Ca^{2+} entry through voltage-dependent channels.

The selectivity shown by laudanosine on NA-induced contraction in KBS was checked in experiments carried out in a Ca^{2+} -free medium. In these conditions, papaverine inhibited the contractile response induced by NA or caffeine, thus confirming its non-specific relaxant activity. Furthermore, when this alkaloid was added during the loading period that permits the refilling of intracellular Ca^{2+} -stores previously depleted by NA or caffeine, posterior addition of these agonists does not induce any contractile response. This indicates that stores cannot be refilled in the presence of papaverine.

Results were different with laudanosine: this alkaloid inhibits NA-induced contractile responses in Ca^{2+} -free medium, but this effect is not attributable to direct inhibition of the smooth muscle contractile elements or release of the intracellular Ca^{2+} -stores because the same concentration of alkaloid did not inhibit caffeine-induced contraction. This confirms the selectivity of laudanosine as an α -adrenoceptor blocking agent. Moreover, laudanosine does not inhibit the

refilling of intracellular stores sensitive to NA or caffeine, which suggests a lack of activity on Ca^{2+} -entry pathways depending on previous depletion of internal Ca^{2+} pools.

Results obtained in the functional study suggest that the inhibitory effect of laudanosine on NA-induced contraction may be due to antagonism of α_1 -adrenoceptors or inhibition of receptor-mediated signal transduction. The relaxation induced by this alkaloid on KCl-induced contraction in the presence of phentolamine suggests a direct inhibition of calcium entry as other benzylisoquinolines does (Ivorra *et al.*, 1992a,c). In order to ascertain whether there is an interaction between laudanosine and the α_1 -adrenoceptor or a receptor site at the Ca^{2+} channel, we studied this possible interaction by radioligand binding techniques. The results obtained indicate that laudanosine interacts with [^3H]-prazosin and [^3H]-(+)-*cis*-diltiazem binding to rat cerebral cortex, although it showed a higher affinity for the α_1 -adrenoceptor binding site than for the benzothiazepine receptor site. Laudanosine does not interact with the [^3H]-nitrendipine binding site.

Analysis of the action of laudanosine on the [^3H]-prazosin binding site, shows that the K_i is in the same range as the IC_{50} obtained from concentration-response curves of laudanosine on NA-induced contraction. These findings provide us with experimental evidence that laudanosine acts as an α_1 -adrenoceptor antagonist with a lower action as calcium entry blocker. Papaverine showed similar specificity to act by both mechanisms.

It is known that the mechanism of the relaxant action of papaverine is related to inhibition of PDEs. In order to determine whether inhibition of PDE is involved in the relaxant response to laudanosine, we tested the effect of this compound on PDE activities isolated from bovine aorta.

There are several molecular forms of PDE identified in smooth muscle. In vascular smooth muscle, four types of cyclic nucleotide phosphodiesterases (PDE) have been isolated: types I and V, which selectively hydrolyze cyclic GMP, and types III and IV, with a high affinity for cyclic AMP (Lugnier *et al.*, 1986; Lindgren *et al.*, 1990; Komasa *et al.*, 1991; Ivorra *et al.*, 1992b). Papaverine is a relatively non-selective inhibitor of these PDE forms and our results show that laudanosine does not exhibit any potent inhibitory action on the different PDE forms differing from papaverine. Present findings clearly suggest that laudanosine has a relaxant effect on rat aorta, which is mediated by interaction with the α_1 -adrenoceptor and the benzothiazepine receptor site at the Ca^{2+} channel, but the inhibition of PDE shown by other benzylisoquinolines like papaverine does not in this case explain the relaxation induced by laudanosine. We must therefore conclude that there are differences between the mechanisms of the relaxant action of the two alkaloids in spite of their structural similarity.

In this context, previous studies (Lugnier *et al.*, 1992; 1994) tested different benzylisoquinoline derivatives as inhibitors of the three PDE forms, and the results obtained indicated that papaverine and its analogues (Compound 1, Table 4) containing an unsaturated heterocyclic ring are the most potent inhibitors of all PDE-forms. The presence of a tetrahydroisoquinoline ring (Compound 4 and laudanosine) implies a loss of activity as PDE-inhibitors. This different behaviour can be explained on the basis of their structural features. The benzyltetrahydroisoquinoline (BTHIQ) alkaloids such as laudanosine, have an sp^3 -like hybridized nitrogen atom, a partially flexible tetrahydroisoquinoline ring and a chiral centre. Papaverine, a benzylisoquinoline (BIQ) has an sp^2 -like nitrogen atom, a planar isoquinoline ring and no chiral centre. The benzyl-dihydroisoquinolines (BDHIQ) analysed have an sp^2 -like nitrogen like papaverine, a partially unsaturated isoquinoline ring and no chiral centre (3,4-dihydropapaverine) or just one (compound 2). These structural features define the geometry of the molecules and can explain the existence of different types of interactions between these agents and the receptor site in the different PDE forms.

Table 4 illustrates how the presence of a chiral centre leads to a loss of effect of the compounds tested on all PDE-forms. The planarity of the isoquinoline ring facilitates the interaction with receptor sites because the more active compounds were benzyloisoquinolines with a totally or partially unsaturated heterocyclic ring (benzyloisoquinolines or benzyldihydroisoquinolines). The different position of the benzyl group does not modify the activity unless this difference determines the presence of a chiral centre (compound 2).

These results provide us with information about a group of compounds that with small structural changes showed a

selective action on each PDE-form isolated from vascular smooth muscle. Further investigations using these compounds could identify the physiological role of each PDE-form in smooth muscle contractile process.

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