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Relationships between structure and vascular activity in a series of benzylisoquinolines

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1 In the present work, the properties of 3-methyl isoquinoline, 3,4-dihydropapaverine, tetrahydropapaverine and tetrahydropapaveroline were compared with those of papaverine and laudanosine. The work includes functional studies on rat isolated aorta contracted with noradrenaline, caffeine or KC1, and a determination of the affinity of the compounds for α_1-adrenoceptors and calcium channel binding sites, with [^{3}H]-prazosin, [^{3}H]-nitrendipine and [^{3}H]-(+)-*cis*-diltiazem binding to rat cerebral cortical membranes. The effects of papaverine derivatives on the different molecular forms of cyclic nucleotide phosphodiesterases (PDE) isolated from bovine aorta were also determined.

2 The three papaverine derivatives show greater affinity than papaverine for the [^{3}H]-prazosin binding site. They are therefore more selective as inhibitors of [^{3}H]-prazosin binding as opposed to [^{3}H]-(+)-*cis*-diltiazem, while papaverine appears to have approximately equal affinity for both. [^{3}H]-nitrendipine binding was not affected by either papaverine or papaverine derivatives in concentrations up to 100 μM. 3-Methylisoquinoline had no effect on any of the binding sites assayed.

3 Contractions evoked by noradrenaline (1 μM) in rat aorta were inhibited in a concentration-dependent manner by 3,4-dihydropapaverine, tetrahydropapaverine and with a lower potency, by tetrahydropapaveroline. In Ca^{2+}-free solution, tetrahydropapaverine and to a lesser extent, tetrahydropapaveroline, inhibited the noradrenaline (1 μM) evoked contraction in a concentration-dependent manner and did not modify the phasic contractile response evoked by caffeine (10 mM). This suggests that these alkaloids do not act at the intracellular level, unlike papaverine which inhibits the contractile response to caffeine and noradrenaline.

4 Inositol phosphates formation induced by noradrenaline (1 μM) in rat aorta was inhibited by tetrahydropapaverine (100 μM) and tetrahydropapaveroline (300 μM), thus suggesting that α_{1D}-adrenoceptors are coupled to phosphoinositide metabolism in rat aorta.

5 Unlike papaverine, which has a significant effect on all the PDE isoforms, the three alkaloids assayed did not have an inhibitory effect on the different forms of PDE isolated from bovine aorta.

6 These results provide evidence that papaverine derivatives with a partially or totally reduced isoquinoline ring have a greater affinity for α_1-adrenoceptors and a lower affinity for benzothiazepine sites in the Ca^{2+}-channel than papaverine. This structural feature also implies a loss of the inhibitory activity on PDE isoforms. The planarity of the isoquinoline ring (papaverine) impairs the interaction with the α_1-adrenoceptor site and facilitates it with the Ca^{2+}-channels and PDEs, whereas the more flexible tetrahydroisoquinoline ring increases the binding to α_1-adrenoceptors.

Keywords: Benzylisoquinolines; α_1-adrenoceptor subtypes; rat aorta; structure-activity relationship; phosphodiesterases

Introduction

Papaverine, a well known smooth muscle relaxant agent, is a benzylisoquinoline alkaloid with multiple activities as a cyclic nucleotide phosphodiesterase (PDE) inhibitor, Ca^{2+}-channel blocker via specific binding to the benzothiazepine receptor site in the Ca^{2+}-channel and α-adrenoceptor antagonist activity (Lacroix *et al*., 1991; Ivorra *et al*., 1992b; Chuliá *et al*., 1994). Because of these multiple mechanisms it is useful as a non-specific spasmolytic agent but cannot be put to more specific use in cardiovascular diseases. However, if we take the chemical structure of papaverine as a model, we may find that small structural differences could lead to more useful compounds with a more definite mechanism of action as PDE-inhibitors, Ca^{2+}-channel blockers or as α-adrenoceptor antagonists.

In order to find out more about the structural requirements that determine greater specificity for one or another of the mechanisms cited above, we have studied a series of papaverine derivatives that are closely related to it structurally. The compounds studied were a simple isoquinoline, 3-methylisoquinoline and three benzylisoquinoline derivatives, one of which had a partially unsaturated isoquinoline ring (3,4 dihydropapaverine); the other two had a tetrahydroisoquinoline ring and were differentiated by the presence (tetrahydropapaveroline) of hydroxyl groups instead of the methoxy groups present in tetrahydropapaverine and papaverine (Figure 1).

We analysed the interaction of the compounds with Ca^{2+}-channels or α-adrenoceptors by examining their effects on [^{3}H]-nitrendipine, [^{3}H]-(+)-*cis*-diltiazem and [^{3}H]-prazosin binding to rat cerebral cortical membranes. We investigated the relaxant action of these compounds on vascular smooth muscle by doing functional studies in rat isolated aorta contracted by addition of noradrenaline or caffeine. We studied the activity of these compounds on inositol phosphate production induced by noradrenaline in rat aorta, and examined their ability to inhibit separate cyclic nucleotide phosphodiesterase enzymes isolated from bovine aortic smooth muscle as papaverine does.

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Some of these data have been presented previously in an abstract form (Garcia *et al*., 1996).

Methods

Radioligand binding experiments

Membranes were prepared from the cerebral cortex of Wistar rats as previously described (Ivorra *et al*., 1992b). Briefly, the tissue was homogenized in 10 vol (w/v) of ice-cold buffer (50 mM Tris-HCl, pH 7.5) with an ultra-turrax (two times, 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 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 [^{3}H]-prazosin (0.1–0.2 nM) or [^{3}H]-nitrendipine (0.3–0.4 nM) or in 50 mM Tris-HCl pH 7.5, containing 1 mg ml^{-1} of bovine serum albumin (BSA) with [^{3}H]-(+)-*cis*-diltiazem (3–4 nM), in the absence or the presence of drugs at various concentrations. Incubations were carried out at 25°C for 30 min ([^{3}H]-prazosin), 90 min ([^{3}H]-nitrendipine) or 120 min ([^{3}H]-(+)-*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) with ice-cold buffer. 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 (1 μM), nifedipine (1 μM) or diltiazem (10 μM), respectively. All results were obtained in triplicate. Displacement curves were analysed by the weighted least-squares iterative curve fitting programme LIGAND (Munson & Rodbard, 1980).

Functional study

Wistar rats of either sex, weighing 200–220 g, were decapitated and the thoracic aorta was isolated. The connective tissue was removed and 5 mm long rings were mounted horizontally under isometric conditions in a 10 ml organ bath containing Krebs-bicarbonate solution (KBS), maintained at 37°C and gassed with 95% O_2 and 5% CO_2. An initial load of 1 g was applied and maintained throughout a 75–90 min equilibration period. This pretension was kept constant, but there was a loss of tension (< 10–15%) when the preparations were placed in Ca^{2+}-free medium. Tension was recorded on a polygraph (Grass M7) via force-displacement transducers (Grass FT03).

KBS had the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 1.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. Ca^{2+}-free solution had the same composition except that $CaCl_2$ was omitted and EDTA (0.1 mM) was added.

Endothelium-denuded aortic rings were prepared by rubbing the entire intimal surface. The absence of relaxant response (100%) after acetylcholine (100 μM) addition to preparations contracted with noradrenaline (1 μM) indicated the absence of a functional endothelium in all the rings (Furchgott & Zawadzki, 1980).

Two different experimental procedures were performed: (i) concentration-response curves of relaxation (CRCR) to alkaloids were obtained by addition of cumulative concentrations of the compounds to aortic rings in which sustained contractions had been induced by 1 μM noradrenaline. Relaxations are expressed as a percentage of the maximum increment in tension obtained by agonist addition. (ii) Figure 2a shows the experimental procedure designed to study the action of alkaloids on the contractile response to noradrenaline in Ca^{2+}-free medium. In order to clarify the intracellular action of the compounds similar experimental procedures were performed but noradrenaline was replaced by caffeine (Figure 2b). Since in Ca^{2+}-free medium, caffeine induced a contraction at 25°C but not at 37°C, the temperature was decreased to 25°C in the protocols in which caffeine was used as an agonist.

Noradrenaline 1 μM was added in Ca^{2+}-containing solution at 37°C and then the tissue was treated with Ca^{2+}-free, EDTA-containing solution for 20 min. After this time noradrenaline 1 μM or caffeine 10 mM was applied and the amplitude of the resulting contraction was monitored as a reference. The tissue was incubated for 20 min in Krebs to refill the intracellular Ca^{2+} stores and a spontaneous increase in the resting tone of the aorta (IRT) was observed only after noradrenaline treatment. After washing and 20 min of loading in Ca^{2+}-free so-

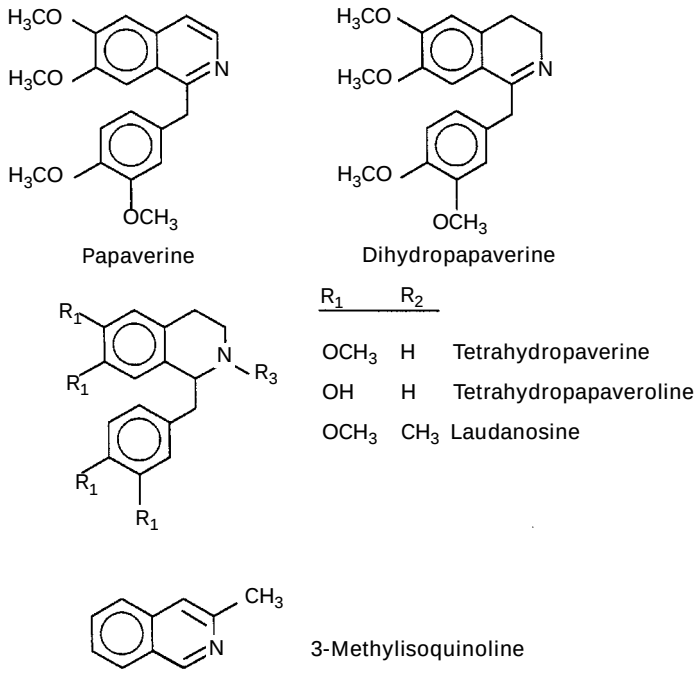

Figure 1 Chemical structures of 2-methyl-isoquinoline, papaverine, dehydropapaverine, tetrahydropapaverine, tetrahydropapaveroline and laudanosine.

lution, noradrenaline or caffeine was added again in the absence of Ca^{2+}; a response similar to the first one obtained in Ca^{2+}-free solution was observed. The effect of each alkaloid added 15 min before was tested on this last agonist-induced contraction (NA_2 or CAF_2 in Figure 2). Following incubation in Krebs for 20 min the addition of agonist allowed the state of the preparation to be checked.

The results are presented as the mean ± s.e. mean for *n* determinations obtained from different animals. E_{max} represents the maximal relaxation (CRCR) obtained after addition of the highest concentration of each compound. The concentration needed to produce 50% relaxation (IC_{50}) was calculated from a linear regression analysis of all the points between 20% and 80% of the maximal response (Graph Pad Software; San Diego, California, U.S.A.)

Inositol phosphate determination

The determination of total inositol phosphate accumulation was adapted from Berridge *et al.* (1982) and Irvine *et al.* (1985). Briefly, rat thoracic aortae (8 animals were killed) were exposed to Krebs Henseleit (KH) containing 2 μCi of myo-[2-^{3}H]-inositol ml^{-1} buffer for 4 h at 37°C and gassed with 95% O_2 plus a 5% CO_2 mixture. After this incubation, the tissue was washed twice with 45 ml Krebs-Henseleit (KH) buffer. Each aorta was cut into four rings which were placed in four individual tubes containing 1 ml KH buffer. This procedure was repeated with all the rat aortae to obtain finally four individual tubes with eight different samples (0.1 – 0.2 g) which were incubated at 37°C for 30 min. LiCl (10 mM) was added 30 s before noradrenaline (1 μM) or buffer solution (control) in order to inhibit the metabolism of inositol monophosphates (Berridge *et al.*, 1982). Tissues were then incubated with saline or alkaloid for 15 min before stimulation. The samples were stimulated for 15 min with noradrenaline (1 μM) in the absence or presence of the alkaloid. Stimulation was stopped by placing the tissue in a cold water bath (4°C) and adding 1.5 ml of a cold mixture of chloroform/methanol/HCl 10 N (100 : 200 : 4, v/v/v) with vigorous shaking. The samples were centrifuged (4000 × g) for 15 min at 4°C. The aqueous phases were brought to pH 4 with 50 ml of ammonium formate 1.2 M and stocked at −20°C until analysis.

The composition of the KH was (mM): NaCl 119, KCl 5.4, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.

The separation of inositol phosphates was performed by a modified method from Irvine *et al.* (1985), with a high performance liquid chromatography (h.p.l.c.) ion-exchange system and a 0.46 cm × 25 cm Partisil SAX 10 high pressure anion exchange column (Shandon, Cergy-Pontoise, France), flow rate 1.3 ml min^{-1}. The aqueous samples (0.5 ml) were loaded onto the column. Distilled water was then allowed to flow for 6 min to elude inositol, and over 24 min a linear gradient was passed through the column rising from 0% to 100% of buffer B (potassium phosphate 1.0 M buffered for 3.7 with orthophosphoric acid). Radioactivity was detected by a Flow-One on-line radioactivity flow detector (Packard, Meriden, USA) equipped with a 2 ml liquid flow cell. Retention times were 15 and 18 min for inositol 1, 4, 5-monophosphate (IP_1) and IP_2, respectively. In this tissue, IP_3 was not detected after a 15 min stimulation period.

The areas of the reconstructed peaks expressed in d.p.m. (sensitivity 1500 d.p.m. mmCi) were related to the total [^{3}H]-inositol incorporated into the tissue. Inhibitions induced by alkaloids were expressed as a percentage of the maximal [^{3}H]-inositol phosphates accumulation induced by noradrenaline (1 μM) incubation in the absence of alkaloid. All values in the text are expressed as the means ± s.e.mean of five or more preparations (*n*) obtained from different animals.

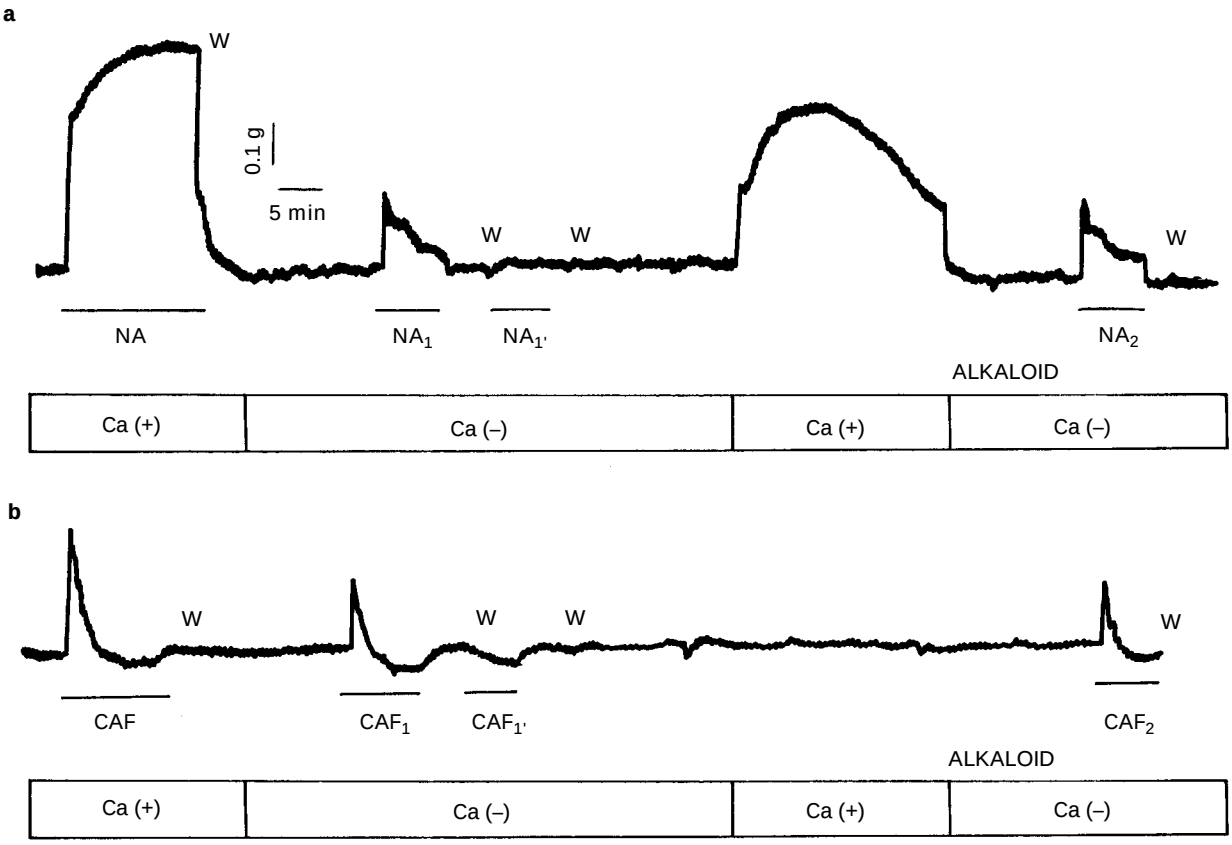

Figure 2 Experimental procedure used to study the effect of alkaloids on noradrenaline-induced (NA) contraction (a) or caffeine-induced (CAF) contraction (b) in the absence of extracellular Ca^{2+}. NA_1 or CAF_1: addition of the agonist after 15 min incubation in Ca^{2+}-free medium. $NA_{1'}$ or $CAF_{1'}$: second addition of the agonist after washing (W) in Ca^{2+}-free medium. NA_2 or CAF_2: addition of the agonist in the presence or absence of the alkaloid (100 mM), after 20 min resting period in KBS (Ca_2+ 1.8 mM) and 15 min in Ca^{2+}-free solution.

Phosphodiesterase inhibition

Cytosolic cyclic nucleotide phosphodiesterase activities (PDE) were isolated form 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 (250 mM saccharose; 25 mM phosphate pH 6.6; 2 mM magnesium acetate (MgAc); 1 mM dithiothreitol; 5 mM ethylene glycol *bis* (b-aminoethylether) N,N,N′,N′ tetraacetic acid (EGTA); 2000 u ml^{-1} aprotinin; 10 $\mu g\ ml^{-1}$ soya bean trypsin inhibitor and 10 $\mu g\ ml^{-1}$ leupeptin) by an ultraturrax (six times, 10 s) and a glass pestle homogenizer, and then centrifuged at 105,000 *g* for 60 min. The resulting supernatant 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 guanosine 3′:5′-cyclic monophosphate (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, dialysed 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 as previously described by Keravis *et al*. (1980) at a substrate ([^{3}H]-cyclic AMP or [^{3}H]-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 $CaCl_2$ and 18 nM calmodulin or in the absence of Ca^{2+} and calmodulin but with 1 mM EGTA. To prevent the influence of cross-contamination between CGI-PDE (PDE 3) and cyclic AMP-PDE (PDE 4), the studies performed with these forms were always carried out in the presence of 50 μM rolipram or 100 μM cyclic GMP, respectively.

The IC_{50} (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 with three different enzymatic preparations.

Data analysis

Where ANOVA showed significant differences ($P<0.05$) the results were further analysed by Student-Newman Keuls test and differences were considered significant when $P<0.05$.

Drugs and solutions

The following drugs were used: acetylcholine chloride, anhydrous caffeine, (-)-noradrenaline bitartrate, papaverine hydrochloride, tetrahydropapaverine hydrochloride, tetrahydro papaveroline hydrobromide (Sigma, St Louis MO, U.S.A.); phentolamine mesylate (RBI, Natick MA, U.S.A.); [^{3}H]-prazosin (72–78 Ci $mmol^{-1}$), [^{3}H]-nitrendipine (70 Ci $mmol^{-1}$), [^{3}H]-(+)-*cis*-diltiazem (154 Ci $mmol^{-1}$), *myo*-[2-^{3}H]-inositol with PT6–271 (10–20 Ci $mmol^{-1}$) (Amersham International, Buckinghamshire, U.K.); [8-^{3}H]-cyclic AMP (30–50 Ci $mmol^{-1}$) and [8-^{3}H]-cyclic GMP (5–15 Ci $mmol^{-1}$) (New England Nuclear, U.K.).

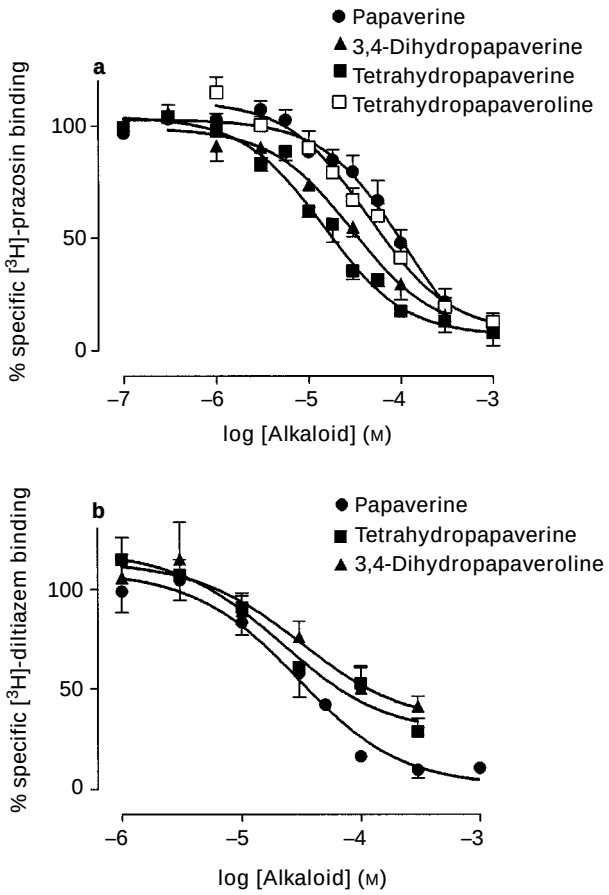

Figure 3 Displacement of the specific binding of [^{3}H]-prazosin (a) or [^{3}H]-(+)-*cis*-diltiazem (b) to the rat cerebral cortex by tetrahydropapaverine, tetrahydropapaveroline, 3,4-dihydropapaverine or papaverine. Each point represents means of 3–8 experiments performed in triplicate with s.e.mean shown by vertical lines.

Table 1 Inhibition by different alkaloids of [^{3}H]-prazosin binding and [^{3}H]-(+)-*cis*-diltiazem binding to specific receptors of rat cerebral cortex membranes

	[^{3}H]-prazosin		*[^{3}H]-(+)-cis-diltiazem*		
Drug	K_i (μM)	*Hill slope*	K_i (μM)	*Hill slope*	*Ratio*
Papaverine	38.6 ± 5.7 ($n=3$)	1.01 ± 0.07	33.6 ± 8.7 ($n=3$)	1.45 ± 0.31	0.870
Dihydropapaverine	18.3 ± 1.6* ($n=3$)	0.86 ± 0.2	104.0 ± 43.7* ($n=4$)	0.84 ± 0.27	5.683
Tetrahydropapaverine	7.9 ± 1.1* ($n=8$)	1.03 ± 0.06	102.0 ± 51.7* ($n=5$)	1.04 ± 0.52	12.879
Tetrahydropapaveroline	18.2 ± 1.11* ($n=4$)	0.90 ± 0.21	ND ($n=2$)	–	–
Laudanosine[1]	5.9 ± 0.7* ($n=3$)	0.95 ± 0.04	26.3 ± 4.5 ($n=3$)	0.91 ± 0.13	4.458

[1]Data from Chuliá *et al*. (1994). ND = no displacement was observed. Ratio = K_i [^{3}H]-(+)-*cis*-diltiazem/K_i [^{3}H]-prazosin. *$P<0.001$ vs papaverine.

Other reagents were of analytical grade. All compounds were dissolved in deionized water except caffeine, which was dissolved in Ca^{2+}-free KBS and tetrahydropapaveroline, which was dissolved in ascorbic acid to a final concentration of 0.001% in order to avoid a possible oxidation of the alkaloid. All solutions were prepared daily and the pH was adjusted to 7.

Results

Binding assays

Binding of [^{3}H]-prazosin to rat cerebral cortex was specific, saturable and showed high affinity. Nonlinear regression analysis of the saturation data was consisted with the presence of a single population of sites. The derived K_m and B_{max} values were 0.11 ± 0.02 nM and 132.5 ± 7.2 fmol mg^{-1} protein, respectively (Sallés & Badía, 1994). [^{3}H]-diltiazem bound to a single class of binding sites in rat cortical membranes homogenate with K_d and B_{max} values of 50 ± 10 nM and 270 ± 30 fmol mg^{-1} protein, respectively (Schaeffer *et al.*, 1988). The specific binding of [^{3}H]-prazosin at a concentration of 0.2 nM and [^{3}H]-diltiazem at a concentration of 3 nM represented approximately 90% and 70% of the total binding, respectively.

The interactions of alkaloids with [^{3}H]-prazosin and [^{3}H]-(+)-*cis*-diltiazem binding are shown in Figure 3. 3-Methylisoquinoline did not inhibit the [^{3}H]-prazosin or [^{3}H]-(+)-*cis*-diltiazem binding to membranes of the rat cerebral cortex. Papaverine and derivatives inhibited [^{3}H]-prazosin binding to cortical membranes (Figure 3a) with an inhibition constant summarized in Table 1. The pseudo-Hill coefficient (slope factor) was not significantly different from unity suggesting direct competition between alkaloids and the radioligand for a single common binding site. In addition, all the papaverine derivatives except tetrahydropapaveroline also inhibited binding of [^{3}H]-(+)-*cis*-diltiazem (Figure 3b). Papaverine inhibited both [^{3}H]-prazosin and [^{3}H]-(+)-*cis*-diltiazem binding in a concentration-dependent manner and gave similar K_i values (Table 1). The three benzylisoquinoline derivatives appeared to be more potent inhibitors of [^{3}H]-prazosin binding and less potent inhibitors of [^{3}H]-(+)-*cis*-diltiazem than papaverine. Tetrahydropapaverine was the most potent inhibitor of the [^{3}H]-prazosin binding (Table 1). [^{3}H]-nitrendipine binding was not affected by either papaverine or papaverine derivatives in concentrations up to 100 μM (results not shown).

Functional study

The magnitude of the sustained contractile response of rat aorta elicited by noradrenaline 1 μM in KBS at 37°C was 876.4 ± 21.7 mg ($n = 8$). This concentration of noradrenaline has proved to be maximal for contraction in rat aorta (Noguera & D'Ocon, 1992).

Adding cumulative concentrations of tetrahydropapaverine (0.1 – 100 μM) produced concentration-dependent relaxations of noradrenaline-precontracted rat aorta (Figure 4). 3,4-Dihydropapaverine showed a slightly lower ability to relax noradrenaline-induced contractions than tetrahydropapaverine or tetrahydropapaveroline (Table 2).

After 15 min in Ca^{2+}-free solution, addition of noradrenaline (1 μM) induced a phasic contraction (187.5 ± 12.8 mg, $n = 10$) followed by a tonic one (89.5 ± 9.9 mg, $n = 10$) (NA_1, see experimental procedure in Figure 2a). After a loading period (20 min) in KBS to refill the intracellular Ca^{2+}-stores, a

Table 2 Inhibitory potencies (IC_{50}, μM) of agents on contractions induced by noradrenaline 1 μM in Ca^{2+}-containing solution (NA-Ca^{2+}(+)) or in Ca^{2+}-free solution (NA-Ca^{2+}(–)) in rat aorta

	NA-Ca^{2+} (+)	*NA-Ca^{2+} (–)*
Papaverine	3.70 ± 1.01 ($n = 5$)	3.81 ($n = 4-6$)
Dihydropapaverine	176.7 ± 41.0* ($n = 3$)	> 100 ($n = 4$)
Tetrahydropapaverine	30.0 ± 3.7* ($n = 5$)	43.92 ($n = 5-6$)
Tetrahydropapaveroline	325.5 ± 88.6* ($n = 4$)	> 300 ($n = 5$)
Laudanosine[1]	11.5 ± 2.3* ($n = 5$)	

[1]Data from Chuliá *et al.* (1994). *$P < 0.001$ vs papaverine.

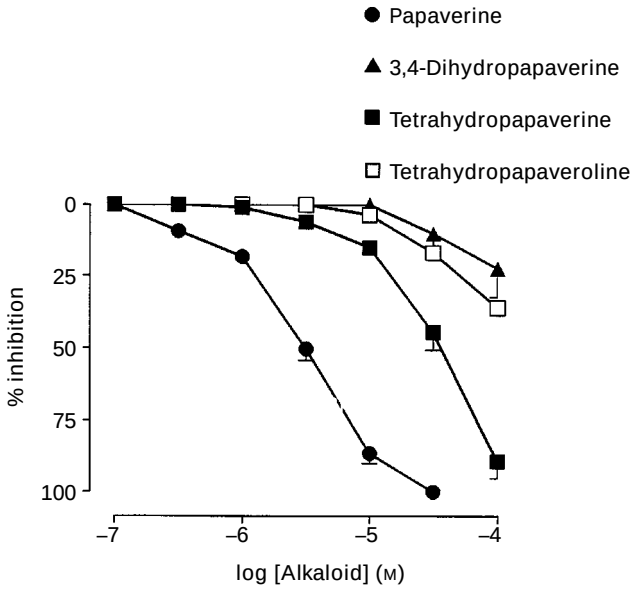

Figure 4 Concentration-response curves of relaxation obtained by addition of different alkaloids to rat aorta precontracted by 1 μM noradrenaline. Each point is the mean derived from n experiments with s.e.mean showed by vertical lines.

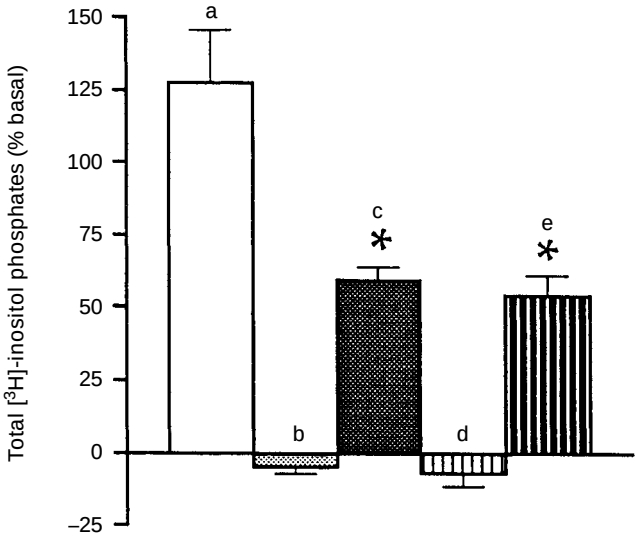

Figure 5 Total [^{3}H]-inositol phosphates production in rat aorta induced by noradrenaline 1 μM in absence (a) or presence of tetrahydropapaverine 100 μM (c) or tetrahydropapaveroline 300 μM (e). (b) and (d) [^{3}H]-inositol phosphates production obtained after treatment with tetrahydropapaverine 100 μM and tetrahydropapaveroline 300 μM alone, respectively. Results are expressed as mean ± s.e.mean from five experiments. *$P < 0.001$, significant inhibition of total [^{3}H]-inositol phosphates accumulation.

Table 3 Effects of agents on cyclic nucleotide phosphodiesterases isolated from bovine aorta

	CaM-PDE PDE 1	*CyclicAMP-PDE PDE 3*	*CyclicAMP-PDE PDE 4*	*CyclicGMP-PDE PDE 5*
Papaverine[1]	27.5 ± 2.2	0.76 ± 0.12	1.68 ± 0.25	1.08 ± 0.36
Tetrahydropapaverine	(11%)	(2%)	(49%)	(36%)
Tetrahydropapaveroline	(0%)	(6%)	(34%)	(24%)

Data shown are mean ± s.e.mean from three determinations obtained on different enzymatic preparations. K_i values were calculated from IC_{50} values according to Cheng and Prusoff (1973) with K_m values for PDE1 = 0.4 μM, PDE3 = 0.4 μM, PDE4 = 1.5 μM, PDE5 = 0.22 μM. Phosphodiesterase activity was assessed with 1 μM [^{3}H]-cyclicGMP + 1 mM EGTA or 1 μM [^{3}H]-cyclicGMP + 10 μM $CaCl_2$ + 18 nM calmodulin for CaM-PDE (PDE 1); 1 μM [^{3}H]-cyclicGMP + 1 mM EGTA for cyclic GMP-PDE (PDE 5); 1 μM [^{3}H]-cyclicAMP + 1 mM EGTA and in the presence of 50 μM rolipram for CGI-PDE (PDE 3) or 100 μM cyclicGMP for cyclicAMP-PDE (PDE 4) in order to limit the cross-contamination of these forms. Numbers in parentheses indicate % inhibition of the enzyme at the drug concentration of 1 mM. [1]Data from Ivorra *et al.* (1992b).

second addition of noradrenaline in Ca^{2+}-free solution (NA_2) induced a similar response in the control experiments but a lower response in the presence of papaverine and tetrahydropapaverine (0.1 – 100 μM). The IC_{50} values obtained for the different alkaloids are shown in Table 2. The maximal concentration assayed of tetrahydropapaveroline and 3,4-dihydropapaverine (100 μM) only inhibited the response to noradrenaline in Ca^{2+}-free medium by 18.1 ± 6.7% ($n = 4$) and 39.6 ± 1.0% ($n = 4$), respectively. Therefore, concentration-response curves for inhibition were not drawn.

At 25°C, caffeine 10 mM induced a rapid transient contraction in Ca^{2+} 1.8 mM solution (342.1 ± 22.4 mg, $n = 15$). Addition of caffeine after 15 min of exposure to a Ca^{2+}-free solution yielded a phasic contraction (121.4 ± 9.7 mg, $n = 15$) (CAF1, Figure 2b). After a loading period (20 min) in KBS to refill the intracellular Ca^{2+}-stores, the caffeine-induced contraction in Ca^{2+}-free medium was restored (CAF_2, Figure 2b) in the control experiments. Preincubation (10 min) with a concentration of 100 μM of each papaverine derivative (tetrahydropapaverine, 3,4-dihydropapaverine and tetrahydropapaveroline) did not modify the phasic contraction induced by caffeine in Ca^{2+}-free medium ($n = 5$ for each alkaloid tested), whereas papaverine, at the same concentration, decreased this contraction (Chuliá *et al.*, 1994).

Effect of tetrahydropapaverine and tetrahydropapaveroline on phosphoinositide metabolism

Figure 5 shows the effect in rat aorta of tetrahydropapaverine (100 μM) and tetrahydropapaveroline (300 μM) on phosphoinositide accumulation induced by noradrenaline (1 μM) in normal KH. The concentrations of tetrahydropapaverine (100 μM) and tetrahydropapaveroline (300 μM) were choosen on the basis of the results obtained in binding experiments.

Our results show that noradrenaline induced a significant increase (127.6 ± 18.0%) in total [^{3}H]-inositol phosphates accumulation relative to the basal value ($n = 5$), which was markedly inhibited by treatment with both alkaloids. The phosphoinositide accumulation induced by noradrenaline 1 μM in the presence of tetrahydropapaverine 100 μM was 49.27 ± 6.64% ($n = 5$) of that induced by noradrenaline alone ($P < 0.001$). Similarly, tetrahydropapaveroline 300 μM significantly inhibited this response, which reached only 44.03 ± 7.65% (n = 4) of the phosphoinositide accumulation induced by noradrenaline ($P < 0.001$).

Inhibition of bovine aorta cyclic nucleotide phosphodiesterase enzymes

We examined the inhibitory effect of tetrahydropapaverine and tetrahydropapaveroline on the different cytosolic molecular forms of PDE isolated from bovine aorta. These were: a PDE form which preferentially hydrolyzes cyclicGMP and is activated by calmodulin (CaM) called CaM-PDE (PDE 1); a cyclic GMP selective form (cyclicGMP-PDE, PDE 5) insensitive to the stimulant effect of CaM; and two PDE forms that specifically hydrolysed cyclicAMP with a low K_m and were not stimulated by the addition of calcium-CaM. One belongs to the cyclicGMP-inhibited family (CGI-PDE, PDE 3) and the other was a rolipram sensitive form (cyclicAMP-PDE, PDE 4) (Lugnier *et al.*, 1986; Ivorra, *et al.*, 1992a).

As shown in Table 3 papaverine exerted a relatively non-selective inhibitory effect on all PDE forms although the inhibition of the calmodulin-sensitive form appeared weaker than that of the other forms. In contrast, the papaverine derivatives did not have a significant effect as inhibitors of the different forms of PDEs assayed.

Discussion

Papaverine is a potent vasorelaxant benzylisoquinoline alkaloid that reduces the contractile response to excitatory agonists (Tashiro & Tomita, 1970; Ferrari, 1974; Chuliá *et al.*, 1994; Aoki *et al.*, 1994). Its action is mainly ascribed to inhibition of cyclicAMP-phosphodiesterases and, therefore, to inducing the accumulation of cytosolic cyclic nucleotides that are identified as intracellular messengers for smooth muscle relaxation. Moreover, recent findings suggest that papaverine relaxes vascular smooth muscle by a mechanism related to a decrease in $[Ca^{2+}]_i$, not only inhibiting Ca^{2+}-influx through voltage-dependent or receptor-operated Ca^{2+}-channels (Lacroix *et al.*, 1991; Chuliá *et al.*, 1994), but also by inhibiting agonist-induced intracellular Ca^{2+}-release by interference with the signal transduction pathway (Aoki *et al.*, 1994). Moreover, the $[Ca^{2+}]_i$-sensitivity of certain contractile mechanisms may be only minimally decreased by papaverine (Aoki *et al.*, 1994). Papaverine has also been shown to have affinity at the [^{3}H]-prazosin binding site in rat cerebral cortex, acting as an α_1-adrenoceptor antagonist (Ivorra *et al.*, 1992b).

These multiple actions of papaverine justify its use as a non-specific relaxant and permit us to analyse the structural requirements that determine higher or lower selectivity of action on each mechanism cited above by a series of different benzylisoquinoline alkaloids.

The results of our previous work made it possible to determine that compounds structurally related to papaverine but containing a tetrahydroisoquinoline ring instead a isoquinoline one, exhibit a more specific activity as smooth muscle relaxants, acting mainly on the Ca^{2+} influx from the extracellular medium, as calcium channel blockers or α_1-adrenoceptor antagonists, but without producing changes in the intracellular distribution of this ion, and with a lower activity as phosphodiesterase inhibitors (Cortes *et al.*, 1990; D'Ocon *et al.*, 1991; Anselmi *et al.*, 1992; Ivorra *et al.*, 1992b; Chuliá *et al.*, 1994). Continuing this line of work we have now studied different compounds structurally related to papaverine but with a different degree of saturation of the isoquinoline ring, in order to determine their selectivity as α_1-adrenoceptor antagonists, Ca^{2+}-channel blockers or phosphodiesterase inhibitors.

In order to ascertain whether there is an interaction between the papaverine derivatives and the α_1-adrenoceptor or a re-

ceptor site at the Ca^{2+} channel, we studied this possible interaction by radioligand binding techniques. The results obtained indicate that all the compounds tested, except 3-methylisoquinoline, displace [^{3}H]-prazosin binding to rat cerebral cortex, and all the compounds tested, except 3-methylisoquinoline and tetrahydropapaveroline, also displace [^{3}H]-(+)-*cis*-diltiazem to rat cerebral cortex. None of them interact with the [^{3}H]-nitrendipine binding site. These data can be interpreted as suggesting that a benzylisoquinoline structure is indispensable and an isoquinoline is not sufficient, to bind to α_1-adrenoceptors and to the benzothiazepine receptor site at the Ca^{2+}-channel. Analysis of the results obtained show that the K_i of papaverine is in the same range for both radioligands as has previously been shown (Chuliá *et al*., 1994) according with the lack of specificity that characterize this alkaloid. As compared to papaverine, the compounds with a dihydroisoquinoline ring (3,4-dihydropapaverine) or tetrahydroisoquinoline ring (tetrahydropapaverine and tetrahydropapaveroline) showed a greater potency as inhibitors of [^{3}H]-prazosin binding and a lower potency as inhibitors of [^{3}H]-(+)-*cis*-diltiazem binding. All of them showed a higher affinity for the α_1-adrenoceptor than for the benzothiazepine binding site in the Ca^{2+}-channel.

These results demonstrate that compounds containing an unsaturated heterocyclic ring have a non-specific mechanism of action, but the presence of a totally or partially saturated tetrahydroisoquinoline or dihydroisoquinoline ring increases the selectivity for the α_1-adrenoceptor and decreases the capacity of binding to the Ca^{2+}-channel and PDE inhibition. Similar results have previously been obtained with another benzyltetrahydroisoquinoline, laudanosine (Chuliá *et al*., 1994).

The different behaviour of the saturated compounds and papaverine at the α_1-adrenoceptors present in rat cerebral cortex can be explained by their structural features. Papaverine has an sp^2-like nitrogen atom and a planar and rigid isoquinoline ring. 3,4-Dihydropapaverine also has an sp^2-like nitrogen but its dihydroisoquinoline ring is not totally planar; and tetrahydropapaverine and tetrahydropapaveroline have an sp^3-like nitrogen atom, a partially flexible tetrahydroisoquinoline ring and a chiral centre. These structural features define the geometry of the molecules and according to our results, determine the greater affinity for the α_1-adrenoceptor. The compound with a tetrahydroisoquinoline ring and all the hydroxyl groups methylated, -i.e. tetrahydropapaverine-, showed the greatest affinity. If we compare the affinity of this compound and another benzyltetrahydroisoquinoline alkaloid, laudanosine, which differs structurally only in the presence of an N-methyl group, no difference can be observed with respect to α_1-adrenoceptor affinity (Table 1).

In the rat cerebral cortex membranes the prazosin-high affinity sites have been demonstrated to be composed of α_{1A} and α_{1B} subtypes at a ratio of approximately 30:70 (Sallés & Badia, 1994; Madrero *et al*., 1996). Our results show that the alkaloids tested were unable to discriminate between α_1-adrenoceptor subtypes, for the binding of [^{3}H]-prazosin was monophasically inhibited by these compounds with Hill slopes not significantly different from unity. The affinity for the two subtypes is, therefore, similar.

In order to ascertain whether these affinities at α_1-adrenoceptors can be explained in terms of an agonist or antagonist activity, we assayed the action of the alkaloids on contractions of the rat aorta which are mediated by: (1) α_1-adrenoceptor activation (noradrenaline-induced contraction in Ca^{2+}-containing or Ca^{2+}-free solution); (2) release of intracellular Ca^{2+} (EDTA- resistant response to caffeine).

Papaverine, 3,4-dihydropapaverine and tetrahydropapaverine relaxed the contractile responses of rat aorta to noradrenaline in KBS, but tetrahydropapaveroline was less effective. The activity of the alkaloids on the noradrenaline-induced response was also tested in a Ca^{2+}-free medium. In this experimental procedure, noradrenaline induces a biphasic contractile response mediated by intracellular Ca^{2+}-release from internal stores (Noguera & D'Ocon, 1992). Preincubation with different concentrations of each alkaloid inhibited this response in a concentration-dependent manner. The IC_{50} obtained was similar to that observed against noradrenaline-induced contraction in Ca^{2+}-containing solution. Tetrahydropapaveroline was also less effective as an inhibitor of noradrenaline-induced contraction in Ca^{2+}-free medium.

Similar experiments were performed with caffeine as the agonist, which elicits a phasic response in Ca^{2+}-free medium. This response was only inhibited by papaverine (Chuliá *et al*., 1994), the other alkaloids tested in the present work did not inhibit the response to caffeine. The fact that the same concentrations of each compound that inhibit noradrenaline-induced contraction did not inhibit the caffeine contraction suggests that the inhibitory effects of the alkaloids on noradrenaline are not attributable to direct inhibition of the smooth muscle contractile elements or release of the intracellular Ca^{2+}-stores. Only papaverine showed a non-specific intracellular action, probably related to its capacity to inhibit phosphodiesterases in smooth muscle.

We also tested the effect of the papaverine derivatives on phosphodiesterase activities isolated from bovine aorta. In vascular smooth muscle, four types of cyclic nucleotide phosphodiesterases have been isolated: PDE 1 and PDE 5, which selectively hydrolyze cyclic GMP, and PDE 3 and PDE 4, with high affinity for cyclic AMP (Lugnier *et al*., 1986; Lindgren *et al*., 1990). Papaverine is a relatively non-selective inhibitor of these phosphodiesterase forms but our results show a lower activity of tetrahydropapaverine and tetrahydropapaveroline with respect to papaverine. The results obtained in the present work corroborate previous observations (Lugnier *et al*., 1994; Chuliá *et al*., 1994) that compounds containing an unsaturated heterocyclic ring are the most potent as inhibitors of the different vascular PDEs (papaverine, Table 3) whereas the presence of a tetrahydroisoquinoline ring (tetrahydropapaverine or tetrahydropapaveroline) leads to the loss of activity as an PDE-inhibitor. The lack of an inhibitory activity on phosphodiesterases justify the results obtained in functional studies, in which tetrahydropapaverine and tetrahydropapaveroline did not show any inhibitory activity as relaxants of caffeine-induced contractions in Ca^{2+}-free medium, unlike other phosphodiesterase inhibitors such as papaverine (Chuliá *et al*., 1994) and IBMX (results not shown).

These results provide us with information about the greater selectivity of action of saturated benzylisoquinolines acting as inhibitors of α_1-adrenoceptors. In order to analyse the transduction mechanisms involved in this inhibitory action on α_1-adrenoceptors, we determined the effect of tetrahydropapaverine and tetrahydropapaveroline on phosphoinositide metabolism. Analysis of the formation of [^{3}H]-inositol phosphates induced by noradrenaline in rat aorta shows that, at concentrations that relax the noradrenaline-induced contraction of rat aorta, both benzyltetrahydroisoquinolines are able to inhibit the phosphoinositol formation pathway linked to α_1-adrenoceptor activation.

These data support the hypothesis that these compounds act as α_1-adrenoceptor antagonists in rat aorta, despite of the lack of correlation between functional studies in this tissue and binding studies in rat cerebral cortex with [^{3}H]-prazosin. We can explain this controversial result if we assume that the population of rat aorta α_1-adrenoceptors is predominantly not the same subtype as the rat cerebral cortex.

The α_1-adrenoceptor subtype predominant in the rat aorta has been classified as the α_{1D}-adrenoceptor (Aboud *et al*., 1993; Ko *et al*., 1994; Kenny *et al*., 1995; Hieble *et al*., 1995). The functional role of the α_{1D}-adrenoceptor has not been established, but in rat aorta it could be related to phosphoinositide hydrolysis, as suggested by Ko *et al*. (1994). The fact that the potency of substances as inhibitors of noradrenaline-induced contraction in rat aorta correlates well with their impotency as inhibitors of phosphoinositide metabolism suggests that the same subtype of adrenoceptor is involved in both processes and, also, implies that α_{1D}-adrenoceptors may cause phos-

phoinositide hydrolysis in rat aorta. The present results support the idea that the three alkaloids tested show a greater and selective affinity for the α_{1A}- and α_{1B}-adrenoceptor subtypes present in rat cerebral cortex, and can discriminate between both these subtypes and the α_{1D}-adrenoceptor present in rat aorta. As affinity values for these compounds on rat aorta adrenoceptors are not available at present, further experiments are needed to confirm this hypothesis.

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