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Multiple actions of glaucine on cyclic nucleotide phosphodiesterases, α_1-adrenoceptor and benzothiazepine binding site at the calcium channel

[1]M. Dolores Ivorra, *Claire Lugnier, *Christa Schott, Maria Catret, M. Antonia Noguera, Elsa Anselmi & Pilar D'Ocon

Laboratori de Farmacognosia i Farmacodinamia, Departament de Farmacologia, Facultat de Farmacia, Universitat de Valencia, Spain and *Université Louis Pasteur de Strasbourg, Laboratoire de Pharmacologie Cellulaire et Moléculaire, Faculté de Pharmacie, CNRS URA 600, BP 24, 67401 Illkirch, France

1 In the present study, the properties of glaucine (an aporphine structurally related to papaverine) were compared with those of papaverine, diltiazem, nifedipine and prazosin. The work includes functional studies on rat isolated aorta contracted with noradrenaline, caffeine or KCl, and a determination of the affinity of glaucine at calcium channel binding sites of α-adrenoceptors, by use of [^{3}H]-(+)-*cis*-diltiazem, [^{3}H]-nitrendipine and [^{3}H]-prazosin binding to cerebral cortical membranes. The effects of glaucine on the different molecular forms of cyclic nucleotide phosphodiesterases (PDE) isolated from bovine aorta were also determined.

2 Contraction evoked by noradrenaline (1 μM) or depolarizing solution (60 mM KCl) were inhibited in a concentration-dependent manner by all the compounds tested. As expected, prazosin showed a greater selectivity of action on NA-induced contraction, whereas nifedipine and diltiazem appeared more potent on KCl-induced contraction. Glaucine had a greater potency on the contraction elicited by noradrenaline whereas papaverine acted non specifically.

3 In Ca^{2+}-free solution, prazosin (0.1 μM) and glaucine (0.1 mM) inhibited the contraction evoked by NA; diltiazem (0.1 mM) diminished this contraction whereas nifedipine (1 μM) had no effect. Preincubation of tissues with glaucine, diltiazem, nifedipine and prazosin did not modify the contractile response induced by caffeine. In contrast, papaverine (0.1 mM) significantly inhibited the contractions evoked by NA or caffeine in Ca^{2+}-free medium.

4 Glaucine and papaverine show affinity at the [^{3}H]-prazosin binding site and at the benzothiazepine binding site of the Ca^{2+}-channel receptor complex, but have no effect at the dihydropyridine binding site in rat cerebral cortex. Glaucine exerts some selectivity as an inhibitor of [^{3}H]-prazosin binding as opposed to [^{3}H]-(+)-*cis*-diltiazem binding while papaverine appears to have approximately equal affinity in this respect.

5 This study confirms the presence of four phosphodiesterase (PDE) activities in bovine aorta: a calmodulin-activated PDE (CaM-PDE type I) which hydrolyzed preferentially guanosine 3':5'-cyclic monophosphate (cyclic GMP); a cyclic GMP selective form (cGMP-PDE type V); and two low K_m adenosine 3':5'-cyclic monophosphate (cyclic AMP) PDEs that are insensitive to the stimulatory effect of CaM, one of which was inhibited by cyclic GMP (CGI-PDE, type III) and the other by rolipram (cAMP-PDE, type IV). Glaucine selectively inhibits one of the two forms of Ca^{2+}-independent low K_m cAMP-PDE, the type IV. In contrast, papaverine exerts a non-selective inhibitory effect upon all PDE forms.

6 The present work provides evidence that glaucine, a benzyltetrahydroisoquinoline alkaloid, has interesting properties as an α_1-adrenoceptor antagonist, calcium entry blocker (through the benzothiazepine recognition site in the calcium channel) and as a selective inhibitor of the rolipram-sensitive cAMP-PDE, type IV PDE.

Keywords: Calcium antagonists; α_1-adrenoceptor blocking agents; rat aorta; glaucine; phosphodiesterase inhibitors

Introduction

Ca^{2+}-entry blockers can be classified into three major subgroups: dihydropyridines, phenylalkylamines and benzothiazepines. In addition, some new structural classes of compounds have recently been reported some of which related to the benzylisoquinoline structure (King *et al.*, 1988; Triggle *et al.*, 1989; D'Ocon *et al.*, 1989; 1991; Lacroix *et al.*, 1991). One of these new compounds is glaucine, an aporphine that is structurally related to papaverine (Figure 1), but shows a mechanism of uterine smooth muscle relaxant activity which is different from papaverine.

The action of papaverine is ascribed to inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) phosphodiesterase (Kukovetz & Pöch, 1970; Lugnier *et al.*, 1972; Bolton, 1979; Cumiskey & Feigenson, 1983), but there is evidence that it may also weakly antagonize calcium flux through specific channels (Schneider *et al.*, 1975; Reinhardt *et al.*, 1977). Glaucine, in rat uterus, showed a relaxant activity similar to that of nifedipine (Anselmi *et al.*, 1992), acting by a more specific mechanism related to inhibition of Ca^{2+}-entry through potential operated Ca^{2+} channels and has no effect on Ca^{2+}-release or Ca^{2+}-redistribution from intracellular storage sites.

In the present study, we compared the properties of glaucine with those of: diltiazem and nifedipine, established calcium antagonists; prazosin, an α_1-adrenoceptor antagonist; and papaverine. The work includes functional studies on rat

[1] Author for correspondence.

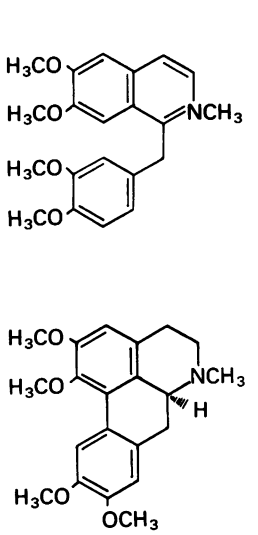

Figure 1 Chemical structures of (a) papaverine and (b) glaucine.

isolated aorta contracted by addition of noradrenaline, caffeine or KCl and in order to assess whether glaucine interacts directly with calcium channel binding sites or, α-adrenoceptors, we examined the effects of glaucine on [^{3}H]-nitrendipine, [^{3}H]-(+)-*cis*-diltiazem and [^{3}H]-prazosin binding to cerebral cortical membranes.

Since the relaxant action of papaverine is ascribed to inhibition of cyclic AMP-phosphodiesterase, we also determined if glaucine has any effects on cyclic nucleotide phosphodiesterase (PDE) activity. In mammalian cells, several distinct molecular forms of PDE exist (for review see Beavo, 1988; Beavo & Reifsnyder, 1990). Accordingly, we examined the ability of glaucine to inhibit the separated PDE enzymes isolated from bovine aorta.

Methods

Functional study

Helically cut strips of 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% O_2 and 5% CO_2. 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 Phillips recorder (PM 8222) coupled to a Hewlett Packard amplifier (8805D) via force-displacement transducers (Gould Statham UC2).

The composition of KBS was as follows (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-free solution had the same composition except that $CaCl_2$ was omitted and EDTA (0.1 mM) was added.

The absence of a relaxant response after addition of 10^{-4} M acetylcholine to preparations previously contracted with noradrenaline (1 μM) indicated the absence of a functional endothelium (Furchgott & Zawadzki, 1980).

Contractions in KBS were expressed in mg. Dose-response curves of relaxation were obtained by addition of cumulative concentrations of the compounds tested on the sustained contractions induced by NA (1 μM) or KCl (60 mM). Relaxations were expressed as a percentage of the maximum tension obtained by agonist addition. E_{max} represents the maximal relaxation obtained after addition of the highest concentration of each compound tested. A regression of response against $-\log$ [C] of test compound was performed by the least squares method for each preparation. The concentration needed to produce 50% inhibition (IC_{50}) was obtained from the linear regression plot of all points between 20–80%: of the maximal response. Contractile responses in Ca-free KBS are expressed as a percentage of the noradrenaline or caffeine-induced contraction in normal KBS.

Results are expressed as the mean ± s.e.mean of 5 or more preparations (n) obtained from different animals. Statistical significance of differences between the means was assessed by Student's t test for unpaired data. 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) with 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 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 in 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) of ice-cold buffer. For [^{3}H]-(+)-*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 10^{-6} M phentolamine, 10^{-6} M nifedipine or 10^{-5} M diltiazem, respectively. All assays were performed in triplicate.

Displacement curves were analyzed by a computerized non-linear curve-fitting programme and K_i values were calculated using the formula of Cheng & Prusoff (1973).

Phosphodiesterase inhibition

Cytosolic cyclic nucleotide phosphodiesterase activities 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 EGTA; 2000 u ml^{-1} aprotinin; 10 μg ml^{-1} soya bean trypsin inhibitor and 10 μg ml^{-1} leupeptin) with an ultra-turrax (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 DEAE-Sephacel ion-exchange column and first eluted by buffer B (25 mM phosphate pH 6.6; MgAc 2 mM and dithiothreitol 1 mM) until no more 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 CaM-PDE from cGMP-PDE 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 a h.p.l.c. column (TSK-DEAE-5PW), washed for 20 min with elution buffer (20 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-step assay described by Wells *et al.* (1975) 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 (CaM) or in the absence of calcium and CaM but with 1 mM EGTA. To prevent the influence of cross-contamination between CGI-PDE (Type III) and cAMP-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 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.

Drugs and solutions

The following drugs were used: (−)-noradrenaline L-tartrate and acetylcholine (Merck); caffeine, papaverine, glaucine, nifedipine, prazosin, (+)-*cis*-diltiazem, cyclic AMP and cyclic GMP (Sigma Chemical Co); phentolamine methanesulphonate (Ciba-Geigy).

[^{3}H]-prazosin (20.2 Ci mmol^{-1}), [^{3}H]-nitrendipine (70 Ci mmol^{-1}), [8-^{3}H]-cyclic AMP (30–50 Ci mmol^{-1}) and [8-^{3}H]-cyclic GMP (5–15 Ci mmol^{-1}) were purchased from the New England Nuclear. [^{3}H]-(+)-*cis*-diltiazem (154 Ci mmol^{-1}) was obtained from Amersham.

Other reagents were of analytical grade.

Nifedipine was dissolved in ethanol (10^{-2} M) before being diluted in distilled water. Caffeine was dissolved in Ca-free KBS. The other drugs were dissolved in distilled water.

Results

Functional study

Noradrenaline at concentrations of 1 μM produced a sustained contraction (348.8 ± 31.0 mg, $n = 9$) in rat isolated thoracic aorta incubated in KBS. This concentration of noradrenaline has proved to be the maximal stimulus for noradrenaline-induced contraction in rat aorta (Noguera & D'Ocon, 1992). Addition of cumulative doses of glaucine, diltiazem, nifedipine, prazosin and papaverine induced concentration-dependent relaxation (Figure 2a) and the E_{max} and IC_{50} for all the products tested are summarized in Table 1.

Another series of experiments was performed in KBS to determine the blocking effect of each product on Ca^{2+} entry through potential-operated Ca^{2+} channels. The contractile response of rat aorta was elicited by depolarizing solution (KCl 60 mM) at 37°C and its magnitude was 225.2 ± 18.5 mg ($n = 18$). All the compounds tested produced concentration-dependent relaxations in KCl-depolarized rat aorta (Figure 2b). The E_{max}, IC_{50} values and the IC_{50} (KCl/IC_{50}(NA) ratio for each product are shown in Table 1. As expected, prazosin showed a greater selectivity of action on NA-induced contraction, whereas nifedipine and diltiazem appeared to have a more potent effect on KCl-induced contraction than on NA-induced contraction. Glaucine showed greater potency on NA-induced contraction, and papaverine showed no specific relaxant action.

Figure 3 shows the experimental procedure used to study the action of the compounds on noradrenaline-induced contraction in the absence of extracellular calcium. After 20 min incubation in Ca^{2+}-free solution, addition of noradrenaline

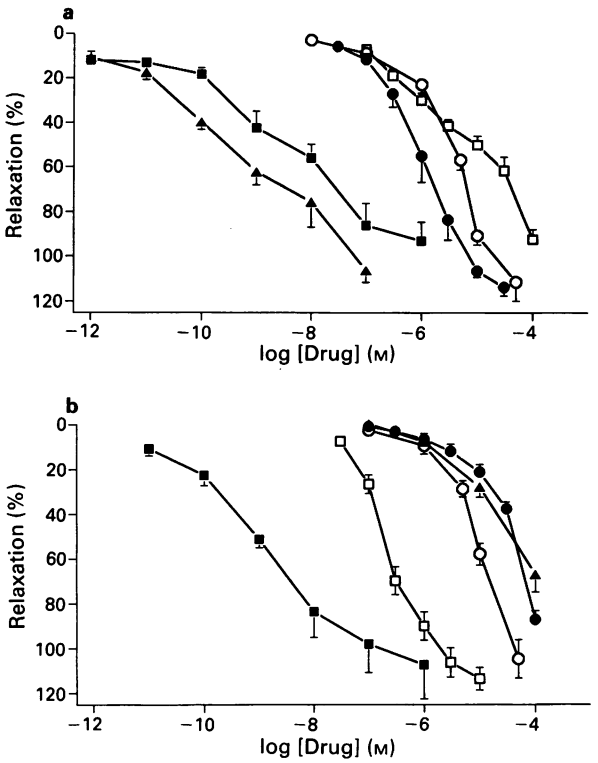

Figure 2 Relaxation dose-response curves obtained after addition of different agents in aorta previously contracted by 1 μM noradrenaline (a) or 60 mM KCl (b). Nifedipine (■); papaverine (○); prazosin (▲); glaucine (●) and diltiazem (□). Each point is the mean derived from n experiments with s.e.mean showed by vertical bars.

(1 μM) induced a phasic contraction, followed by a tonic contraction which is due to release of intracellular calcium (Karakai *et al.*, 1987; Karaki, 1989). The magnitude of this noradrenaline-induced contraction (NA_1) relative to the contraction in the presence of 1.8 mM Ca^{2+} was 24.1 ± 1.5% and 14.9 ± 2.5% ($n = 8$) for the phasic and the tonic step respectively. However the contraction was not evoked when noradrenaline 1 μM was added in Ca^{2+}-free solution a second time (NA_2). After a 20 min resting period in KBS and after 20 min in Ca^{2+}-free solution, addition of NA (NA_3) induced a contraction similar in magnitude to the first one (NA_1), thereby indicating that preincubation with 1.8 mM Ca^{2+} for 20 min restored the calcium at the intracellular storage sites. In order to study the effect of the different agents on this NA-induced contraction in Ca-free medium, the maximal concentrations of compounds that completely inhibit NA-induced contraction in KBS were added 10 min before the addition of NA (NA_3). This addition of NA in Ca^{2+}-free solution did not induce a contractile response in the presence of prazosin (10^{-7} M), papaverine (10^{-4} M) and glaucine (10^{-4} M). A lower response to NA was observed in the presence of diltiazem (10^{-4} M), whereas nifedipine (10^{-6} M) had no effect on NA_3-elicited contraction in Ca^{2+}-free solution (Table 2). These results indicate that only prazosin, papaverine and glaucine block the contractile response due to α-adrenoceptor activation in Ca^{2+}-free medium.

In order to clarify the possible action of glaucine and diltiazem on α-adrenoceptor or on the inhibition of Ca^{2+} release from the intracellular storage sites, similar experimental procedures were used but NA was replaced by caffeine (Caf) and temperature was decreased to 25°C (Figure 3b). In these conditions, Caf 10 mM induced a rapid transient contraction in Ca^{2+} 1.8 mM solution (168.6 ± 13.0 mg, $n = 11$). Addition of Caf_1 after 20 min of exposure to a Ca^{2+}-free solution yielded a phasic contraction with a magnitude that

Table 1 Inhibitory potencies of agents on contractions induced by noradrenaline (NA) and KCl in rat aorta

		NA	*KCl*	$IC_{50(NA)}/IC_{50(KCl)}$
Glaucine	E_{max} (%)	113.8 ± 3.8	87.3 ± 3.9	
		$n = 5$	$n = 11$	
	pIC_{50}	6.08 ± 0.07	4.40 ± 0.04*	0.021
Nifedipine	E_{max} (%)	93.2 ± 8.7	107.3 ± 15.2	
		$n = 5$	$n = 5$	
	pIC_{50}	8.07 ± 0.11	9.16 ± 0.10*	12.30
Diltiazem	E_{max} (%)	92.4 ± 4.5	113.6 ± 5.1	
		$n = 6$	$n = 5$	
	pIC_{50}	5.30 ± 0.12	6.78 ± 0.16*	30.19
Prazosin	E_{max} (%)	108.0 ± 4.1	67.1 ± 7.7	
		$n = 5$	$n = 4$	
	pIC_{50}	9.70 ± 0.09	4.42 ± 0.19*	5.2×10^{-6}
Papaverine	E_{max} (%)	111.5 ± 8.3	104.8 ± 8.5	
		$n = 7$	$n = 6$	
	pIC_{50}	5.51 ± 0.10	5.29 ± 0.07	0.60

$pIC_{50} = (-\log M)$
Values are means ± s.e.mean
*Significant difference ($P < 0.001$) from the corresponding values on NA-induced contraction.

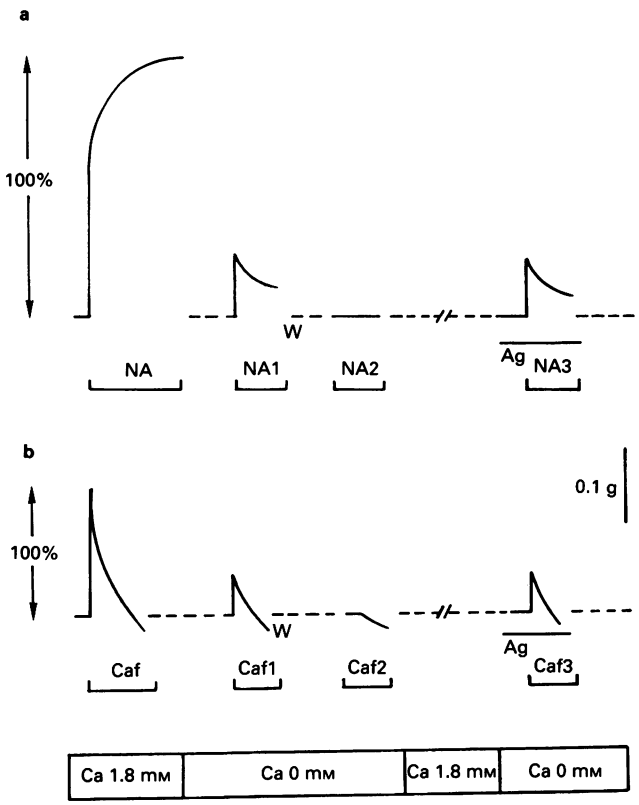

Figure 3 Schematic representation of the effect of the agents on noradrenaline (NA)-induced contraction (a) or caffeine (Caf)-induced contraction (b) in the absence of extracellular calcium. NA_1 or Caf_1: addition of the agonist after 20 min incubation in Ca^{2+}-free medium; NA_2 or Caf_2: second addition of the agonist after washing (W) in Ca^{2+}-free medium; NA_3 or Caf_3: third addition of the agonist after a 20 min resting period in KBS (Ca^{2+} 1.8 mM) and 20 min in Ca^{2+}-free solution. Ag: tested compound.

was 29.4 ± 4.4% ($n = 8$) of the value measured in the presence of Ca^{2+}. After washing, addition of Caf_2 did not induce any contractile response. After a loading period (20 min) in Krebs solution, to refill the intracellular Ca^{2+}-stores, Caf_3-induced contraction in Ca^{2+}-free medium (20 min) was restored. Pre-

Table 2 Effects of agents on noradrenaline (NA)-induced contraction of rat aorta in Ca^{2+}-free medium

Agent	*[C]*	*NA1* (%)	*NA3 + Agent* (%)	n
Glaucine	10^{-4} M	23.96 ± 8.32	—	8
Nifedipine	10^{-6} M	22.12 ± 1.36	20.30 ± 1.60	6
Diltiazem	10^{-4} M	21.76 ± 2.38	4.92 ± 1.36*	5
Prazosin	10^{-7} M	17.19 ± 3.43	—	3
Papaverine	10^{-4} M	26.97 ± 3.40	—	4

[C] Maximal concentration of agent that completely inhibits noradrenaline-induced contraction in Krebs solution.
% are calculated from the initial contraction by noradrenaline in Krebs solution.
Values are mean ± s.e.mean.
n = number of experiments.
*$P < 0.001$ from NA1.

incubation with glaucine (10^{-4} M), diltiazem (10^{-4} M), nifedipine (10^{-6} M) or prazosin (10^{-7} M) did not modify the contractile response induced by Caf_3 in Ca^{2+}-free medium after refilling the intracellular Ca^{2+} stores. Papaverine (10^{-4} M) significantly diminished the phasic contraction elicited by Caf_3 (Table 3).

Binding assays

Binding of [^{3}H]-prazosin to rat cerebral cortex was saturable, reversible and showed high affinity, with a dissociation constant K_d of 0.14 nM (Schott *et al.*, 1988). [^{3}H]-nitrendipine and [^{3}H]-(+)-*cis*-diltiazem also bound to a single class of binding site in rat cortical membrane homogenate, with K_d values of 0.7 nM and 50 nM, respectively (Schaeffer *et al.*, 1988).

The interactions of glaucine and papaverine with [^{3}H]-prazosin and [^{3}H]-(+)-*cis*-diltiazem binding are shown in Figure 4. Glaucine fully inhibited [^{3}H]-prazosin binding to cortical membranes, with an inhibition constant of 0.32 ± 0.03 μM (Figure 4a). The pseudo-Hill coefficient (slope factor) was not significantly different from unity ($n_H = 1.01 \pm 0.13$), which suggests direct competition between glaucine and the radioligand for a single common binding site. In addition, glaucine inhibited binding of [^{3}H]-(+)-*cis*-diltiazem (Figure 4b) but with a lower affinity ($IC_{50} = 10.02 \pm 2.59$ μM) and with a low pseudo-Hill slope ($n_H = 0.73 \pm 0.06$). This indi-

Table 3 Effects of agents on caffeine (Caf)-induced contraction of rat aorta in Ca^{2+}-free medium

Agent	*[C]*	*Caf1* (%)	*Caf3 + Agent* (%)	n
Glaucine	10^{-4} M	28.27 ± 4.87	24.35 ± 5.28	5
Nifedipine	10^{-6} M	30.87 ± 4.28	32.95 ± 4.50	5
Diltiazem	10^{-4} M	33.83 ± 5.50	32.32 ± 1.89	5
Prazosin	10^{-7} M	31.16 ± 6.21	25.19 ± 3.62	4
Papaverine	10^{-4} M	32.96 ± 2.13	19.32 ± 4.72*	8

[C] Maximal concentration of agent that completely inhibits noradrenaline-induced contraction in Krebs solution.
% are calculated from the initial contraction by caffeine in Krebs solution.
Values are mean ± s.e.mean
n = number of experiments.
*$P < 0.002$ from Caf1.

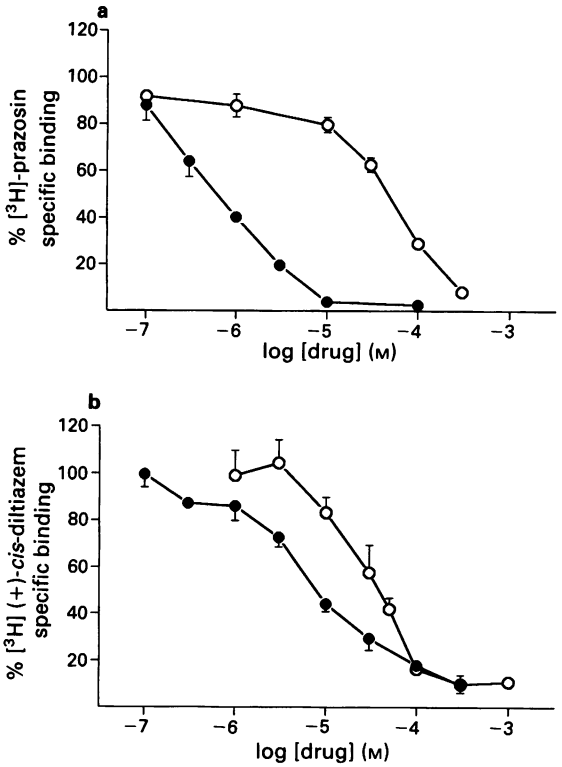

Figure 4 Displacement curves of [^{3}H]-prazosin (a) or [^{3}H]-(+)-*cis*-diltiazem specific binding (b) by papaverine (O) or glaucine (●). Each point is the mean from three experiments done in triplicate with s.e.mean showed by vertical bars.

cates that the interaction between glaucine and [^{3}H]-(+)-*cis*-diltiazem is not a simple competition for a single common binding site but a more complex interaction. Papaverine inhibited both [^{3}H]-prazosin and [^{3}H]-(+)-*cis*-diltiazem binding in a concentration-dependent manner, showing similar K_i values for both radioligands ($K_i = 33.6 \pm 8.8\,\mu M$, $n_H = 1.45 \pm 0.31$ and $K_i = 19.7 \pm 2.6\,\mu M$, $n_H = 0.83 \pm 0.16$, respectively). Glaucine appeared to have more affinity at the [^{3}H]-prazosin and [^{3}H]-(+)-*cis*-diltiazem binding site than papaverine. Moreover, glaucine was approximately 60 times more potent than papaverine in inhibiting [^{3}H]-prazosin binding, but was only 4 times more potent than papaverine in inhibiting [^{3}H]-(+)-*cis*-diltiazem binding.

[^{3}H]-nitrendipine binding was not affected by either papaverine or glaucine in concentrations up to 100 μM (results not shown).

We have also tested the effect of rolipram, a specific inhibitor of PDE type IV, on [^{3}H]-prazosin, [^{3}H]-diltiazem and [^{3}H]-nitrendipine binding to cortical membrane and have observed that rolipram did not modify the binding of these radioligands in concentrations up to 300 μM.

Inhibition of bovine aorta cyclic nucleotide phosphodiesterases

We have examined the inhibitory effect of glaucine and papaverine on the following cytosolic molecular forms of PDE isolated from bovine aorta: a PDE form called CaM-PDE (type I) which preferentially hydrolyzes cyclic GMP and is activated by calmodulin (CaM); a cyclic GMP-selective form (cGMP-PDE; type V) insensitive to the stimulatory effect of CaM; and two PDE forms that specifically hydrolyze cyclic AMP with a low K_m and which are not stimulated by the addition of Ca^{2+}-CaM; one belongs to the cyclic GMP-inhibited family (CGI-PDE, type III) and the other is a rolipram-sensitive form (cAMP-PDE, type IV) (Lugnier *et al.*, 1986; Ivorra *et al.*, 1992).

As shown in Table 4, 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, glaucine proved to be a potent and selective inhibitor of the rolipram-sensitive form of low K_m cAMP-PDE (type IV) with a K_i value of 3.3 μM, whereas it had no effect on the CGI-PDE form ($IC_{50} > 300\,\mu M$). It also inhibited hydrolysis of cyclic GMP by CaM-PDE but with a low potency, exerting similar inhibitory effects both on the basal or the activated (in the presence of calcium and CaM) state of the enzyme (K_i values of 63.3 and 44.4 μM, respectively). In addition, glaucine did not exert any inhibitory effect on the cyclic GMP hydrolytic activity of cGMP-PDE ($IC_{50} > 300\,\mu M$).

Discussion

In previous work we have shown that the relaxant effects of benzylisoquinolines in uterine smooth muscle vary, depending on whether or not an unsaturated heterocyclic ring (papaverine) or a tetrahydroisoquinoline ring (cularines, glaucine, antioquine) was present. This structural feature implies a more specific activity, similar to that of Ca^{2+}-entry blockers (D'Ocon *et al.*, 1989; 1991; Anselmi *et al.*, 1992). This selective action showed by benzyltetrahydroisoquinolines implies an inhibition of Ca^{2+}-influx from the extracellular medium without changes in the intracellular distribution of this ion. In contrast, the benzylisoquinolines with an unsaturated ring induce a relaxation mediated by both a decrease in the Ca-entry through specific channels and a decrease in the intracellular free Ca^{2+}-levels (Cortes *et al.*, 1990; Anselmi *et al.*, 1992).

These findings prompted us to examine the potential activity of the alkaloid glaucine, which shows these structural characteristics, as a Ca-channel blocker. We began the study by analyzing the relaxant action of glaucine on the contractile response of rat aorta induced by noradrenaline or KCl, in order to ascertain whether or not the action of the alkaloid was selective. A comparison of the relaxation induced by glaucine with the relaxant responses elicited by nifedipine, diltiazem, prazosin and papaverine was also made.

Analysis of the IC_{50} (NA)/IC_{50} (KCl) ratios provides information on selectivity (Table 1) and indicates that glaucine, like prazosin, exhibits greater inhibition of the contractile response induced by NA than KCl. As expected, nifedipine and diltiazem show more potent relaxant effects on responses to KCl, whereas papaverine relaxes contractile responses to NA or KCl in a non-selective way, as described in earlier papers (Bolton, 1979).

The selectivity shown by glaucine on NA-induced contraction in KBS was checked in the experiments carried out in a

Table 4 Effects of agents on cyclic nucleotide phosphodiesterases isolated from bovine aorta

PDE activity	*^{3}H-substrate*	K_i (μM) *Glaucine*	*Papaverine*
CaM-PDE type I	Cyclic GMP + EDTA	63.3 ± 20.6	8
	Cyclic GMP + CaM	44.4 ± 9.8	27.5 ± 2.2*
cGMP-PDE type V	Cyclic GMP + EGTA	> 300	1.08 ± 0.36*
CGI-PDE type III	Cyclic AMP + EGTA	> 300	0.76
cAMP-PDE type IV	Cyclic AMP + EGTA	3.3 ± 0.23	1.68

Data shown are mean ± s.e.mean from three determinations obtained in different enzymatic preparations. K_i values were calculated from IC_{50} values according to Cheng & Prusoff. PDE activity was assessed with 1 μM of [^{3}H]-cyclic GMP + 1 mM EGTA or 1 μM [^{3}H]-cyclic GMP + 10 μM $CaCl_2$ + 18 nM calmodulin for CaM-PDE; 1 μM of [^{3}H]-cyclic GMP + 1 mM EGTA for cGMP-PDE; 1 μM [^{3}H]-cyclic AMP + 1 mM EGTA and in the presence of 100 μM of rolipram for CGI-PDE or 100 μM of cyclic GMP for cAMP-PDE type IV in order to limit the cross-contamination of these forms.
*Data from Lugnier *et al.* (1986).

nominally Ca^{2+}-free medium. Under these conditions, NA and Caf induce a contractile response that is due only to the release of intracellular Ca^{2+} from the internal stores sensitive to the agonists. The mechanism whereby NA and Caf cause Ca^{2+}-release is not entirely known, but the fact that prazosin inhibits NA- but not Caf-induced contraction in Ca^{2+}-free medium indicates that the α_1-adrenoceptor is directly related to this Ca^{2+}-release induced by NA, probably by release of 1,4,5-inositol trisphosphate.

In contrast, papaverine inhibits the contractile response to both NA and Caf in these experimental procedures, again confirming its non-specific relaxant activity.

While nifedipine does not modify the contraction induced by NA or Caf in Ca^{2+}-free medium, diltiazem partially diminishes the NA-elicited contraction but produces no changes in the contractile response to Caf.

Glaucine completely inhibited NA-induced contractile responses in Ca-free medium and this effect is not attributable to direct inhibition of the smooth muscle contractile elements, or release of the intracellular calcium stores because the same concentration of alkaloid did not inhibited Caf-induced contraction. It has been suggested that, in rat aorta, the NA-induced release of intracellular Ca^{2+} is attributable to α_1-adrenoceptor activation (Chiu *et al.*, 1987; Daly *et al.*, 1990) whereas the Caf-induced Ca^{2+}-release is due to a different mechanism (Itoh *et al.*, 1983; Karaki *et al.*, 1987; Sato *et al.*, 1988). Therefore, glaucine can selectively inhibit Ca^{2+}-release mediated by α_1-adrenoceptor activation in rat aorta. This effect may be due to antagonism of α_1-adrenoceptors, or inhibition of receptor-mediated signal transduction. In order to identify the mechanism we studied the interaction of glaucine with the α_1-adrenoceptor, using radioligand binding techniques, and compared these effects with those of papaverine. The results obtained indicate that both glaucine and papaverine interact with [^{3}H]-prazosin binding to rat cerebral cortex although glaucine showed a higher affinity for the α_1-adrenoceptor binding site than did papaverine.

However, besides blocking α_1-adrenoceptors, glaucine may also have Ca^{2+}-entry blocking properties because it antagonizes contractions induced by depolarizing solution as do calcium entry blockers like nifedipine and diltiazem. For this reason, we have evaluated the effect of glaucine and papaverine on [^{3}H]-nitrendipine and [^{3}H]-(+)-*cis*-diltiazem binding sites. Both alkaloids interact with the benzothiazepine binding site of the Ca^{2+}-entry blocker receptor complex but have no effect at the dihydropyridine binding site.

Although both papaverine and glaucine inhibit [^{3}H]-(+)-*cis*-diltiazem and [^{3}H]-prazosin binding, the present results demonstrate that glaucine exerts a measure of selectivity as an inhibitor of [^{3}H]-prazosin binding compared with [^{3}H]-(+)-*cis*-diltiazem binding, while papaverine appears to show approximately equal affinity in this respect. These findings largely agree with the results obtained in the functional studies, where glaucine appeared more potent in relaxing NA-induced contraction than KCl-induced contraction, whereas papaverine relaxed both contractions in a similar manner.

On the other hand, many authors (Kukovetz & Pöch, 1970; Lugnier *et al.*, 1972; Van Inwegen *et al.*, 1979) postulated that the mechanism of action of many isoquinoline derivatives, including papaverine, involves inhibition of PDE. In order to determine to what extent inhibition of PDE may be involved in the vasorelaxant response to glaucine, we tested the effect of this compound on PDE activities isolated from bovine aorta as a model for vascular smooth muscle.

It is now apparent that there are several molecular forms of PDE identified in most mammalian tissues, including smooth muscle. In vascular smooth muscle four types of PDE have been isolated. Type I PDE (CaM-PDE) is Ca^{2+}/camodulin-dependent and preferentially hydrolyzes cyclic GMP, whereas type V (cGMP-PDE), which selectively hydrolyzes cyclic GMP, is not stimulated by Ca^{2+}/calmodulin (Lugnier *et al.*, 1986). Types III and IV are two forms of Ca^{2+}-independent PDE with a high affinity for cyclic AMP; the type III (CGI-PDE) is selectively inhibited by cyclic GMP, cilostamide and some cardiotonic agents, whereas type IV is insensitive to cyclic GMP inhibition but is selectively inhibited by rolipram (Ahn *et al.*, 1989; Lindgren *et al.*, 1990; Komas *et al.*, 1991; Ivorra *et al.*, 1992).

Our results show that glaucine selectively inhibits one of the two forms of Ca^{2+}-independent low K_m cAMP-PDE, type IV, whereas papaverine has an equipotent inhibitory effect on both forms. We have also evaluated the inhibitory effect of glaucine on the other molecular forms of PDE that preferentially hydrolyze cyclic GMP. Glaucine inhibits the CaM-PDE form but the effect is less potent compared with effects on cAMP-PDE type IV and this alkaloid has no effect on cGMP-PDE hydrolytic activity. In contrast, papaverine is a relatively non-selective inhibitor of these PDE forms (Lugnier *et al.*, 1986). The inhibitory effect of glaucine on CaM-PDE was tested both in the presence and absence of Ca^{2+}/calmodulin since the ability of a drug to inhibit the activation of the enzyme by calmodulin can be used as a test system for characterizing possible calmodulin antagonist properties. Since glaucine exerts a similar inhibitory effect on the basal or activated state of the enzyme, we can also exclude the possibility that glaucine has calmodulin-antagonist action.

These data are the first account of a benzylisoquinoline structure showing a selective inhibitory effect on one low K_m cAMP-PDE form (Type IV). There is increasing evidence for selective forms of PDE being involved in controlling different biological processes and thus selective PDE inhibition can lead to a variety of discrete pharmacological responses (Weishaar *et al.*, 1985; Beavo, 1988). In this way, inhibition of the CGI-PDE enzyme present both in cardiac (Harrison *et al.*, 1986; Reeves *et al.*, 1987; Komas *et al.*, 1989) and vascular tissues (Ahn *et al.*, 1989; Lindgren *et al.*, 1990; Komas *et al.*, 1991; Ivorra *et al.*, 1992) is important for cardiotonic/vasodilator activity (Harrison *et al.*, 1986; Kauffman *et al.*, 1987; Weishaar *et al.*, 1987; Kithas *et al.*, 1988; Ivorra *et al.*, 1992)

whereas inhibition of the cAMP-PDE, type IV, an enzyme characterized also in cerebral tissue (Strada *et al.*, 1984; Nemoz *et al.*, 1985) may be important because of its antidepressant properties (Weishaar *et al.*, 1985; Schultz & Schmidt, 1986; Sutor *et al.*, 1990).

On the basis of these findings, it seems unlikely that only the inhibitory effect of glaucine on cAMP-PDE type IV can account for the vasorelaxant properties observed in this functional study.

The different pharmacology exhibited by papaverine and glaucine might be attributed to geometry or to differences in the basicity of the heteroatom. The structural features of glaucine that define its geometry are the presence of an sp^3-like hybridized nitrogen atom, a chiral centre and also a partially flexible tetrahydroisoquinoline ring. These topological characteristics and the electronic characteristics concerning the basicity at the nitrogen site could determine a specific mode of interaction with the α-adrenoceptor, benzothiazepine receptor site at the calcium channel and inhibition of PDE IV.

In summary, the present work provides evidence that the benzyltetrahydroisoquinoline derivative, glaucine, has affinity for [^{3}H]-prazosin and [^{3}H]-diltiazem binding sites which probably account for α_1-adrenoceptor antagonist and calcium antagonist properties (through benzothiazepine receptor site in the calcium channel). The compound also shows selective inhibition of type IV PDE.

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