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A possible structural determinant of selectivity of boldine and derivatives for the α_{1A}-adrenoceptor subtype

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1 The selectivity of action of boldine and the related aporphine alkaloids, predicentrine (9-O-methylboldine) and glaucine (2,9-O-dimethylboldine) on α_1-adrenoceptor subtypes was studied by examining [^{3}H]-prazosin competition binding in rat cerebral cortex. WB 4101 and benoxathian were used as selective α_{1A}-adrenoceptor antagonists.

2 In the competition experiments [^{3}H]-prazosin (0.2 nM) binding was inhibited by WB 4101 and benoxathian. The inhibition curves displayed shallow slopes which could be subdivided into high and low affinity components (pK_i = 9.92 and 8.29 for WB 4101, 9.35 and 7.94 for benoxathian). The two antagonists recognized approximately 37% of the sites with high affinity from among the total [^{3}H]-prazosin specific binding sites.

3 Boldine, predicentrine and glaucine also competed for [^{3}H]-prazosin (0.2 nM) binding with shallow and biphasic curves recognizing 30–40% of the sites with high affinity. Drug affinities (pK_i) at the high and low affinity sites were, 8.31 and 6.50, respectively, for boldine, 8.13 and 6.39 for predicentrine, and 7.12 and 5.92 for glaucine. The relative order of selectivity for α_{1A}-adrenoceptors was boldine (70 fold α_{1A}-selective) = predicentrine (60 fold, α_{1A}-selective) > glaucine (15 fold, α_{1A}-selective).

4 Pretreatment of rat cerebral cortex membranes with chloroethylclonidine (CEC, 10 μM) for 30 min at 37°C followed by thorough washing out reduced specific [^{3}H]-prazosin binding by approximately 70%. The CEC-insensitive [^{3}H]-prazosin binding was inhibited by boldine monophasically (Hill slope = 0.93) with a single pK_i value (7.76).

5 These results suggest that whereas the aporphine structure shared by these alkaloids is responsible for their selectivity of action for the α_{1A}-adrenoceptor subtype in rat cerebral cortex, defined functional groups, namely the 2-hydroxy function, induces a significant increase in α_{1A}-subtype selectivity and affinity.

Keywords: α_1-Adrenoceptor subtypes; [^{3}H]-prazosin binding; rat cerebral cortex; aporphine alkaloids; boldine; predicentrine; glaucine

Introduction

Papaverine, a benzylisoquinoline alkaloid, is a well-known spasmolytic agent that acts by a non-specific inhibition of cyclic nucleotide phosphodiesterases (PDEs) (Lugnier *et al.*, 1972; Bolton, 1979; Cumiskey & Feigenson, 1983; Ivorra *et al.*, 1992); but there is also evidence that it may antagonize Ca^{2+} influx (Reinhardt *et al.*, 1977) and act on α_1-adrenoceptors (O'Hara & Ono, 1986; Ivorra *et al.*, 1992). In previous work we have shown that a series of benzylisoquinoline derivative alkaloids, structurally related to papaverine, have a relaxant effect on the vascular smooth muscle that is also related to their capacity to inhibit Ca^{2+} influx through voltage-operated Ca^{2+} channels, act as α_1-adrenoceptor antagonists and inhibit all or some of the different forms of PDEs isolated from aorta (Ivorra *et al.*, 1992; 1993; Chuliá *et al.*, 1994).

The importance of the results obtained residues in the fact that small changes in the structure of papaverine lead to compounds with a selective action for one or another of the above mentioned mechanisms. It thus seems that compounds with a tetrahydroisoquinoline ring, instead of the isoquinoline ring present in papaverine, act mainly at α_1-adrenoceptors. This is the case for laudanosine, a benzyltetrahydroisoquinoline alkaloid (Chuliá *et al.*, 1994), and for some aporphine structures such as glaucine, boldine and apomorphine (Ivorra *et al.*, 1992; 1993). This more specific action on α_1-adrenoceptors was accompanied by a loss in the activity of these alkaloids as PDEs inhibitors. Radioligand binding studies have shown that, of these compounds, boldine is the most potent in inhibiting [^{3}H]-prazosin binding to rat cortical cerebral membranes, and it seems to exhibit a complex interaction at α_1-adrenoceptors that suggests the presence of more than a single class of binding site (Ivorra *et al.*, 1993).

As α_1-adrenoceptors are not homogeneous and several subtypes have been described (for review see Ford *et al.*, 1994; Hieble *et al.*, 1995; Michel *et al.*, 1995), the above observations prompted us to study the possible subtype selectivity of boldine (Figure 1) for α_1-adrenoceptor subtypes present in rat cerebral cortex. In order to explore some structural requirements of α_1-adrenoceptor selectivity, we also studied the effects of two other aporphines, predicentrine (9-O-methylboldine) and glaucine (2,9-O-dimethylboldine), differing from boldine only by the methylation of one or both phenolic hydroxyl groups (Figure 1). WB4101 and benoxathian were used as selective α_{1A}-adrenoceptor antagonists. The affinities of different compounds for the α_{1A}- and α_{1B}-adrenoceptor subtypes were evaluated by studying their ability to displace specific [^{3}H]-prazosin binding from rat cerebral cortex membranes.

Methods

Binding study

Preparation of membranes Female Wistar rats (180–200 g) were decapitated and the brain was rapidly removed. The cerebral cortex was homogenized in 10 vol.(w/v) of ice-cold buffer (Tris HCl 5 mM, sucrose 250 mM and EDTA 1 mM, pH 7.5 at 25°C) using an ultra-turrax (two, 15 s). The homogenate was centrifuged for 10 min at 1000 *g*. The pellet was discarded

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and the supernatant was centrifuged at 50,000 *g* for 15 min at 4°C. The pellet was resuspended in the same volume of assay buffer (Tris HCl 50 mM, pH 7.5) and centrifuged at 50,000 *g* for 15 min at 4°C. The final pellet was resuspended in assay buffer and stored at −70°C for later use. All procedures to prepare membranes were conducted at 4°C.

For pretreatment with chloroethylclonidine (CEC), aliquots of membranes were incubated for 30 min at 37°C with 10 μM CEC in assay buffer (Tris HCl 50 mM, pH 7.5). The reaction was stopped by dilution with ice-cold buffer, followed by three successive 15 min centrifugations at 50,000 *g* to wash the membranes extensively and completely remove any remaining unbound drug.

[^{3}H]-prazosin binding studies Binding of [^{3}H]-prazosin was measured in aliquots of diluted membranes incubated in 50 mM Tris buffer (pH 7.5) with [^{3}H]-prazosin (0.2 nM) and in the absence or presence of 17–20 concentrations of the indicated agents. The incubation volume was 1 ml (approximately 250 μg protein/tube), but displacement experiments with CEC-treated membranes were carried out in a final volume of 2 ml (approximately 500 μg protein/tube). The assay tubes were incubated for 45 min at 25°C, and then binding reactions were terminated by rapid vacuum filtration using a Brandel cell harvester (M24R) with glass fibre filters (Schleicher and Schuell, No. 30) presoaked in 0.3% polyethylenimine for 5 min. The filters were then washed four times with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.5), and the filter-bound radioactivity was determined by liquid scintillation counting. Nonspecific binding was defined as binding in the presence of 10 μM phentolamine. Assays were conducted in duplicate.

Proteins were assayed according to the method of Bradford with globulin as standard (Bradford, 1976).

Data analysis

Competition binding experiments were analysed by the weighted least-squares iterative curve fitting programme, LIGAND (Munson & Rodbard, 1980). The data were first fitted to a one- and then a two-site model, and if the residual sums of squares were statistically less for a two-site fit of data than for a one-site fit, as determined by an *F*-test comparison, then the two-site model was accepted. The experimental results were expressed as means ± s.e.mean for *n* determinations obtained from different animals. When ANOVA showed significant differences ($P<0.05$) the results were further analysed by the Student-Newman Keuls test. *P* values less than 0.05 were considered significant.

Drugs

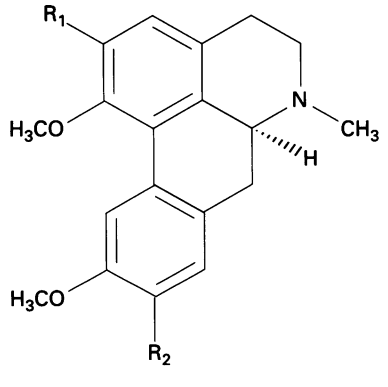
S-(+)-Boldine (Figure 1) was isolated from the plant, *Peumus boldus* in the Departamento de Química de la Universidad de Chile as previously described (Speisky *et al.*, 1991); predicentrine (Figure 1) was prepared from boldine by selective 9-O-methylation (Asensio *et al.*, 1993). The purity of both compounds (>99%) was confirmed by t.l.c. and spectral methods (mass, n.m.r.). S-(+)-Glaucine (Figure 1) was purchased from Sigma Chem. Co. The following drugs (sources in parentheses) were used: [^{3}H]-prazosin (specific activity 72–78 Ci mmol^{-1}, Amersham, U.K.); WB 4101 hydrochloride (2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane), benoxathian hydrochloride, phentolamine mesylate, chloroethylclonidine dihydrochloride (Research Biochemicals Inc, Natick, MA, U.S.A.).

Results

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_d and B_{max} values were 0.11 ± 0.02 nM and 132.5 ± 7.2 fmol mg^{-1} protein, respectively (Sallés & Badía, 1994). Chloroethylclonidine (CEC) has been reported to inactivate selectively the α_{1B}-subtype (Han *et al.*, 1987a). Pretreatment of rat cortical membranes with CEC reduced the density of α_1-adrenoceptors labelled with [^{3}H]-prazosin by 70–75% (Sallés & Badía, 1994) without significantly changing the K_d value for [^{3}H]-prazosin (Kenny *et al.*, 1994).

The specific binding of 0.2 nM [^{3}H]-prazosin to the α_1-adrenoceptors was completely inhibited by WB 4101, benoxathian (Figure 2) and by boldine, predicentrine and glaucine (Figure 3). As expected, displacement curves for WB 4101 and benoxathian were shallow, and computerized analysis of these curves revealed the presence of two distinct sites. The proportion of the high affinity sites defined by the two antagonists amounted to approximately 37% of the total specific binding of [^{3}H]-prazosin (Table 1).

Boldine, predicentrine and glaucine also displayed biphasic curves (Figure 3), and the LIGAND analysis fitted the data to a two site model. From the two site fits we calculated that approximately 30–40% of the sites had high affinity for these



	R_1	R_2
Boldine	OH	OH
Predicentrine	OH	OCH_3
Glaucine	OCH_3	OCH_3

Figure 1 Chemical structure of boldine, predicentrine and glaucine.

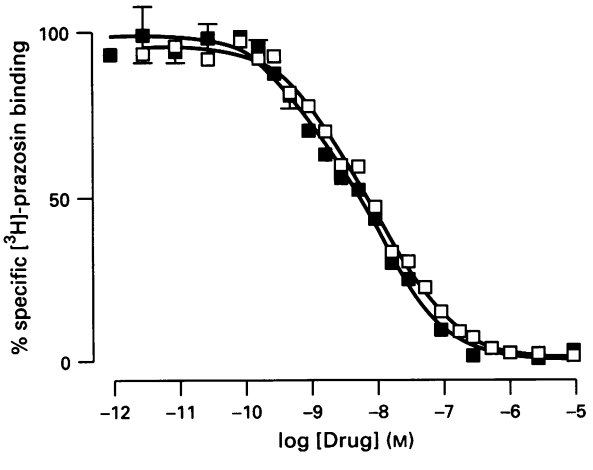


Figure 2 Displacement of specific [^{3}H]-prazosin (0.2 nM) binding from rat cerebral cortex membranes by WB 4101 (■) and benoxathian (□). Values represent the means ± s.e.mean from 3–4 individual experiments performed in duplicate.

compounds (Table 1). This proportion of high affinity sites detected with aporphine alkaloids is similar to that obtained for WB 4101 and benoxathian, and corresponds to the pharmacologically defined α_{1A}-adrenoceptor. In some experiments (4 out of 11), glaucine inhibited [^{3}H]-prazosin binding monophasically (LIGAND analysis fitted the data to a one site model) with a single pK_i value (pK=6.13±0.09, n=4).

Displacement affinities (pK_i) for the high and low affinity sites for the different compounds as well as the affinity ratio between subtypes ($r_{KI/Kh}$) are shown in Table 1. Boldine was the most selective agent for α_{1A}-adrenoceptors and was 70 fold more potent at the α_{1A}- than at the α_{1B}-subtype; predicentrine was 56 fold more potent although there was not a significant difference with respect to boldine; glaucine was only 17 fold more potent. Moreover, in the case of glaucine there is a significant loss of affinity for both α_1-adrenoceptor subtypes with respect to boldine and predicentrine.

Although the antagonists, WB-4101 and benoxathian, exhibited higher affinity for α_1-adrenoceptor subtypes than boldine, they were less selective for the α_{1A}-adrenoceptor subtype and they were found to be only 40 and 22 fold more potent, respectively, at the α_{1A}- than at the α_{1B}-subtype.

The effect of CEC pretreatment on inhibition curves for boldine is shown in Figure 4. CEC has been used because of its alkylating effects, which are selective for α_{1B}-adrenoceptors relative to α_{1A}-adrenoceptors (Han *et al.*, 1987a). As in other studies, pretreatment of rat cortical membranes for 30 min at 37°C with 10 μM CEC produced a significant reduction in the [^{3}H]-prazosin specific binding (the proportion of the remaining sites was 27.6±6.0%, n=5) compared with the binding of the membranes without CEC pretreatment. Total binding and specific binding at 0.2 nM [^{3}H]-prazosin were 3716±441 d.p.m. and 91.7±1.5% in CEC-untreated membranes, and 1101±161 d.p.m. and 74±3.8% in CEC-pretreated membranes. Therefore CEC pretreatment inactivated only a proportion of the α_1-adrenoceptor binding sites labelled with [^{3}H]-prazosin that corresponds to the proportion of α_{1B}-adrenoceptor subtypes determined in the competition experiments in membranes not pretreated with CEC.

The binding of 0.2 nM [^{3}H]-prazosin in CEC-pretreated membranes was monophasically inhibited by boldine (Figure 4) with a Hill slope not significantly different from unity (n_H=0.93±0.08). The pK_i value obtained was 7.76±0.09 (n=5) and was close to the affinity of boldine for the high affinity sites in CEC-untreated membranes (Table 1).

Discussion

Recently, it was found that α_1-adrenoceptors constitute a heterogeneous family of receptors, and several subtypes have been identified by use of a variety of techniques: functional studies, radioligand binding and molecular biology. Although differences in protocol and technique have resulted in several classification schemes, the existence of three subtypes is now recognized, i.e. α_{1A}, α_{1B}, and α_{1D}-adrenoceptors (for review see Ford *et al.*, 1994; Hieble *et al.*, 1995; Michel *et al.*, 1995). Initially, two subtypes of α_1-adrenoceptors (α_{1A} and α_{1B}), were defined on the basis of binding experiments. Binding sites for [^{3}H]-prazosin with high affinity for WB-4101 and phentolamine (Morrow & Creese, 1986), as well as oxymetazoline (Hanft & Gross, 1989a), benoxathian (Han *et al.*, 1987b), 5-methylurapidil (Gross *et al.*, 1988), and S-(+)-niguldipine (Boer *et al.*, 1989) were designated α_{1A}-adrenoceptors, whereas binding sites with low affinity for these ligands were designated α_{1B}-adrenoceptors. In addition, the alkylating agent, chlor-

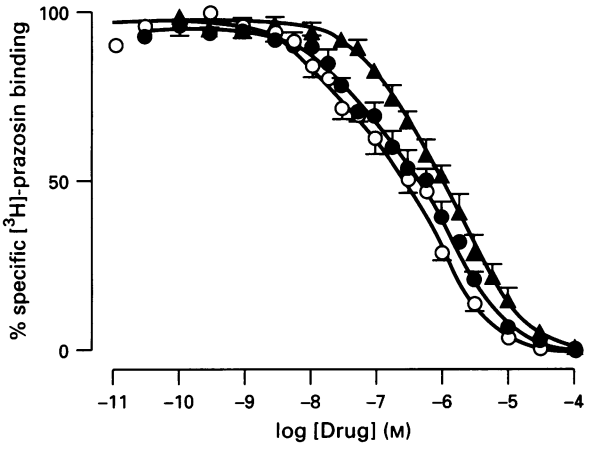


Figure 3 Displacement of specific [^{3}H]-prazosin (0.2 nM) binding from rat cerebral cortex membranes by boldine (○), predicentrine (●) and glaucine (▲). Values represent the means±s.e.mean from 3–7 individual experiments performed in duplicate.

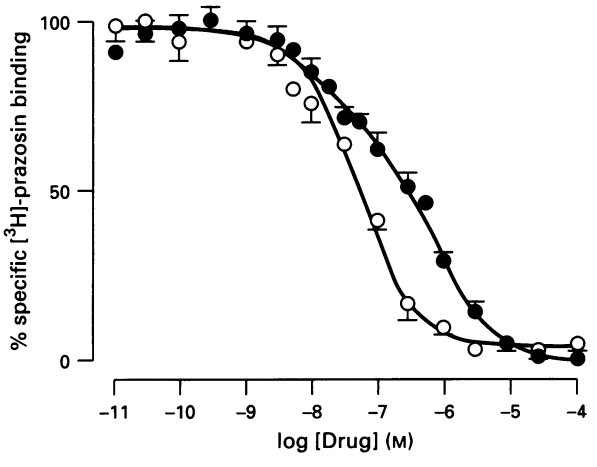


Figure 4 Effect of *in vitro* pretreatment of rat cerebral cortex membranes with chloroethylclonidine (CEC, 10 μM) on the inhibition of specific [^{3}H]-prazosin (0.2 nM) binding by boldine. Displacement experiments were performed in control membranes (●) or in CEC-pretreated membranes (○). Values represent the means±s.e.mean from 3–7 individual experiments performed in duplicate.

Table 1 Inhibition of [^{3}H]-prazosin binding to α_1-adrenoceptors of rat cerebral cortex membranes by the different agents

Drug	n	$pK_{i\ high}$	$pK_{i\ low}$	*%high*	*Hill slope*	$r_{KI/Kh}$
WB4101	4	9.92±0.07	8.29±0.06	37.1±2.6	0.78±0.04	42±2
Benoxathian	3	9.35±0.09	7.94±0.08	37.7±2.6	0.68±0.01	22±2
Boldine	6	8.31±0.05	6.50±0.08	33.2±1.6	0.77±0.03	70±12
Predicentrine	3	8.13±0.10	6.39±0.11	30.9±5.5	0.74±0.05	56±6
Glaucine	7	7.12±0.10**	5.92±0.07*	41.8±3.9	0.83±0.06	17±2*

Displacement experiments were done with 0.2 nM [^{3}H]-prazosin and 17–20 concentrations of competing drug. p$K_{i\ high}$ or p$K_{i\ low}$, negative log of the equilibrium dissociation constant (−log M) at high and low affinity sites for drug tested. %high, population binding at the high affinity site compared to the total specific binding sites. $r_{Ki/Kh}$, ratio between $K_{i\ low}$ and $K_{i\ high}$. Data shown are mean±s.e.mean. n=number of experiments. *P<0.01 *vs* boldine and predicentrine; **P<0.001 *vs* boldine and predicentrine.

oethylclonidine (CEC) has been proposed to inactivate irreversibly α_{1B}- but not α_{1A}-adrenoceptors (Han *et al.*, 1987a; Minneman *et al.*, 1988).

Molecular biological techniques have allowed the cloning and expression of three receptors, i.e., α_{1b} (Cotecchia *et al.*, 1988), α_{1c} (Schwinn *et al.*, 1990) and the α_{1d} (Lomasney *et al.*, 1991; Perez *et al.*, 1991). It has been difficult to establish the relationship between pharmacologically defined and cloned α_1-adrenoceptors, and their classification is still evolving. Until recently only the cloned and pharmacologically defined α_{1B}-adrenoceptors were considered to represent the same molecular entity (Cotecchia *et al.*, 1988; Lomasney *et al.*, 1991). Currently, however, the α_{1c} clone is also considered to be the same receptor as the classical α_{1A}-adrenoceptor (Ford *et al.*, 1994; Faure *et al.*, 1994; Perez *et al.*, 1994; Pimoule *et al.*, 1995; Blue *et al.*, 1995). The cloned α_{1d}-adrenoceptor seems to represent a novel subtype (i.e. the α_{1D} adrenoceptor). Thus, the new nomenclature of α_1-adrenoceptors with high affinity for prazosin (native and recombinant receptors: α_{1A} and α_{1a}, α_{1B} and α_{1b}, α_{1D} and α_{1d}, respectively) was recently acknowledged by IUPHAR (Hieble *et al.*, 1995).

These receptor subtypes have different tissue distributions, and in the rat cerebral cortex membranes the prazosin-high affinity sites have been demonstrated to be composed of α_{1A} and α_{1B} subtypes (Morrow & Creese, 1986; Hanft & Gross, 1989b; Graziadei *et al.*, 1989; Oshita *et al.*, 1991; Sallés & Badia, 1994).

In our study, the competition curves in rat cerebral cortex of the known α_{1A} selective antagonists, WB 4101 and benoxathian for [^{3}H]-prazosin binding were shallow and biphasic and revealed the existence of α_{1A}- and α_{1B}-adrenoceptors at a ratio of approximately 37:63%, which is in good agreement with previously published data (Morrow & Creese, 1986; Hanft & Gross, 1989b; Sallés & Badia, 1994). The affinity constants at their high and low affinity sites are also in accordance with these previous reports.

Like WB 4101 and benoxathian, boldine also bound to [^{3}H]-prazosin high affinity sites in rat cerebral cortex with two different affinities, thus indicating the presence of at least two α_1-adrenoceptor subtypes. Boldine recognized approximately 33% high affinity sites. Since this proportion is similar to the percentage of high affinity sites for WB 4101 and benoxathian, which are the pharmacologically-defined α_{1A}-adrenoceptors, it is likely that boldine high affinity sites are of the α_{1A}-subtype. This implies that boldine is a selective α_{1A}-adrenoceptor agent relative to α_{1B}-adrenoceptors that exhibits a greater selectivity than that of WB 4101 or benoxathian, for boldine is approximately 70 fold α_{1A}-selective, whereas the selectivities of WB or benoxathian are 40 fold and 20 fold, respectively. Boldine, may therefore be a useful tool for studying the α_1-adrenoceptor subtypes present in different tissues.

In order to corroborate the selective action of boldine on the α_{1A}-adrenoceptor subtype, we tested its effect on [^{3}H]-prazosin binding to rat cerebral cortical membranes pretreated with CEC. In rat cortical membranes, the pharmacological profile of CEC-insensitive sites appeared to be indicative of α_{1A}-adrenoceptors, since selective antagonists like WB 4101, benoxathian, phentolamine, niguldipine, or 5-methylurapidil, displayed potencies comparable to that obtained with the high affinity sites in competitive studies in normal membranes (Hanft & Gross, 1989b; Hanft *et al.*, 1989; Han & Minneman, 1991; Sallés & Badia, 1994), and with cloned α_{1A}-receptors (for review see Michel *et al.*, 1995). In these experimental conditions, boldine displayed a steep, monophasic competition curve for [^{3}H]-prazosin binding to rat cortical membranes pretreated with CEC with a single pK_i value which is close to the affinity of boldine for the high affinity sites determined in membranes not pretreated with CEC.

The present results demonstrate the capacity of boldine, an aporphine alkaloid, to discriminate between the two subtypes of α_1-adrenoceptors present in rat cerebral cortex. Functional studies in rat aorta also suggest a subtype selectivity for boldine. Previous results obtained in our laboratory showed that in rat aorta, noradrenaline concentration-response curves in the presence of boldine could not be accounted for by simple competitive antagonism at a single receptor (Schild slopes significantly lower than unity), and this suggests a heterogeneity of α_1-adrenoceptors in rat aorta that could be differentiated by boldine (Ivorra *et al.*, 1993). To date, the data on the α_1-adrenoceptor subtypes present in rat aorta are confusing. Although some studies have suggested that the α_1-adrenoceptor population is a homogeneous entity which has been postulated to be either α_{1A} (Beckering & Brodde, 1989), α_{1B} (Eltze & Boer, 1992; Testa *et al.*, 1995), non-α_{1A}/non-α_{1B} (Oriowo & Ruffolo, 1992; Aboud *et al.*, 1993), α_{1D} (Ko *et al.*, 1994), or 'predominantly' α_{1D} (Kenny *et al.*, 1995), others have suggested that a number of different subtypes contribute to the contractile response in rat aorta (Piascik *et al.*, 1991; Van der Graaf *et al.*, 1996). Moreover, Rokosh *et al.* (1994) and Piascik *et al.* (1994) reported that they could detect mRNA for the α_{1b}, α_{1c} and α_{1d} adrenoceptors in the rat aorta.

In order to identify some structural feature accounting for the selectivity showed by boldine, we have assayed the effect of glaucine, another aporphine alkaloid structurally related to boldine, that also exhibited α_1-adrenoceptor antagonistic activity in different tissues (Ivorra *et al.*, 1992; Orallo *et al.*, 1993; 1995). The binding of [^{3}H]-prazosin to rat cerebral cortex membranes was inhibited biphasically by glaucine, indicating two different sites. However, glaucine not only exhibited a significant loss of affinity for α_1-adrenoceptors relative to boldine, but also a loss of selectivity between α_{1A}- and α_{1B}-subtypes since it is only approximately 15 fold α_{1A}-selective. Moreover, in some competition binding experiments glaucine was unable to discriminate between subtypes probably due to its low selectivity ratio. A non-selective action of glaucine on α_1-adrenoceptors subtypes has previously described in rat vas deferens (Orallo *et al.*, 1993).

Boldine and glaucine have similar structures: they are aporphine alkaloids with the configuration **S** and with oxygenated substituents at the same positions. The only difference is the degree of methylation of oxygen atoms. Boldine has two free hydroxyl groups at C-2 and C-9, whereas glaucine has all the oxygen atoms methylated (Figure 1). The results obtained in the present work showed that the methylation of free hydroxyl functions leads to a drastic decrease in binding affinity and selectivity for α_1-adrenoceptor subtypes. In order to study whether one of the hydroxyl groups may be primarily responsible of this loss of activity we have also analysed the effect of predicentrine, which possesses only a free 2-hydroxyl group. The results obtained showed that predicentrine maintains the ability of boldine to discriminate between the α_{1A} and α_{1B} subtypes with a similar potency and selectivity (60 fold selective for α_{1A}- relative to α_{1B}-adrenoceptors), which suggest that the presence of a free 2-hydroxy function on the (**S**)-aporphine skeleton significantly increases the α_1-subtype selectivity shown by these aporphine alkaloids.

In summary the present study shows that the three (**S**)-aporphine alkaloids tested by us were able to act at α_1-adrenoceptor subtypes with different degrees of affinity and selectivity (boldine = predicentrine > glaucine), thus suggesting that an aporphine structure is responsible for this activity, whereas a defined functional group, namely the 2-hydroxy function, seems to be a critical factor in the ability of these compounds to discriminate between α_1-adrenoceptor subtypes. It is interesting to note that methylation of the hydroxyl group at the 2-position (glaucine) results in a significant loss in potency and selectivity. This kind of compound could be important not only for the development of new selective agents for the α_1-adrenoceptor family, but also a more thorough structure-activity relationship analysis could lead to understanding of the interactions between the ligand and the different α_1-adrenoceptor subtypes.

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