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Vascular Activity of (–)-Anonaine, (–)-Roemerine and (–)-Pukateine, Three Natural 6a(*R*)-1,2-Methylenedioxyaporphines with Different Affinities for α_1-Adrenoceptor Subtypes

Abstract

We have studied the mechanism of action of three 6a(*R*)-1,2-methylenedioxyaporphines as vasorelaxant compounds. The alkaloids assayed showed different affinities for the three human cloned α_1-adrenoceptor (AR) subtypes stably expressed in rat-1 fibroblasts, showing lower affinity for α_{1B}-AR with regard to the α_{1A}- or α_{1D}-subtypes. These three natural compounds are more potent inhibitors of [^3H]-prazosin binding than of [^3H]-diltiazem binding to rat cerebral cortical membranes. As all these alkaloids inhibited noradrenaline (NA)-induced [^3H]-inositol phosphate formation in cerebral cortex and rat tail artery, they may be safely viewed as α_1-AR antagonists, as is demonstrated by the vasorelaxant responses observed in isolated rat tail artery and/or aorta precontracted with NA. The alkaloids also inhibited the contractile response evoked by KCl (80 mM) but with a lower potency than that shown against NA-induced contraction. We have also examined their ability to inhibit the different forms of cyclic nucleotide phosphodiesterases (PDE) isolated from bovine aortic smooth muscle and endothelial cells, with negative results. We conclude that *N*-methylation favours the interaction of *(R)*-aporphines with all α_1-AR subtypes, and that the topography of the binding site recognizing the basic or protonated nitrogen atom is similar in all three α_1-AR subtypes. The presence of a hydroxy group at C-11 has different effects on the affinity for each α_1-AR subtype but decreases the affinity for Ca^{2+} channels. These results confirm and extend the view that subtle changes in the hydroxylation patterns on the aromatic ring of the aporphine structure affect the interactions of these compounds with the three α_1-AR subtypes in different ways, suggesting that the binding site recognizing the aporphine skeleton is different in each of the three subtypes.

Key words

1,2-Methylenedioxyaporphines · human cloned α_1-adrenoceptor subtypes · rat cerebral cortex · rat aorta · rat caudal artery · cyclic nucleotide phosphodiesterases

Abbreviations

PDE:	cyclic nucleotide phosphodiesterase
AR:	adrenoceptor
NA:	noradrenaline
[^{125}I]-HEAT:	[^{125}I]iodo-2[β-(4-hydroxyphenyl)-ethylamino-methyl]-tetralone

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Bibliography

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Introduction

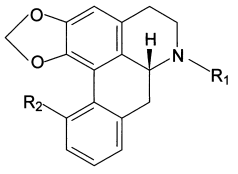
In the last few years a number of reports have shown that aporphine alkaloids exercise a variety of different activities in the cardiovascular system [1], [2], [3], [4], [5]. We have demonstrated that vasorelaxation by different natural and semi-synthetic aporphines is largely due to their α_1-adrenergic antagonism, with a possible lesser component involving Ca^{2+} channel inhibition [6], [7], [8]. Moreover, results obtained from radioligand binding studies provided evidence that a homologous series of 1,2,9,10-tetraoxygenated *6a(S)*-aporphine alkaloids exhibited α_{1A}-adrenoceptor (AR) subtype selectivity [9], [10], [11], whereas *6a*(*R*)-10,11-dihydroxyaporphines showed higher affinity for the α_{1D}-subtype [12]. Finally among the aporphines tested we evidenced that glaucine possesses selective activity for phosphodiesterase type 4 (PDE4) *versus* the other types [6].

In this work, we have extended the study of the vascular activity of aporphine alkaloids to a subset of three natural (*R*)-aporphines (Fig. **1**) bearing a methylenedioxy group at the 1,2-position. We have determined the affinity of these compounds for the three human cloned α_1-AR subtypes (α_{1A}, α_{1B} and α_{1D}) [13] and for native α_1-AR present in rat cerebral cortical membranes (α_{1A}-, and α_{1B}-AR) [9]. Their affinity for Ca^{2+} channels was also assessed using membranes prepared from rat cerebral cortex. The functional activity of these compounds was evaluated by analyzing their effect on the second messenger system associated with α_1-AR stimulation (inositol phosphate formation), as well as their vasorelaxant activities against noradrenaline- (NA) or KCl-induced contraction. Finally, their ability to inhibit the different forms of cyclic nucleotide phosphodiesterase (PDE) was also studied.

Materials and Methods

Animals

Female Wistar rats (200 – 220 g) were bred in a standard experimental animal room of the Faculty of Pharmacy. The rats were housed under a 12-h light/dark cycle at 22 °C and 60% humidity. All experimental procedures were approved by the Experimental Animal Ethical Committee of the Faculty of Pharmacy of the University of Valencia (Spain).



R_1	R_2	
H	H	*(R)*(-)-Anonaine
CH_3	H	*(R)*(-)-Roemerine
CH_3	OH	*(R)*(-)-Pukateine

Fig. **1** Chemical structures of the tested compounds.

Stable expression of human α_1-AR subtypes in rat-1 fibroblasts

Transfection of each cDNA into rat-1 fibroblasts was accomplished by using the calcium phosphate precipitation method as described previously [14]. Individual colonies were isolated, subcloned and screened for high expression (1 – 2 pmol mg^{-1} of total protein). Transfected rat-1 fibroblasts stably expressing human α_{1A}-, α_{1B}- or α_{1D}-AR were grown and selected according to Schwinn et al. [14].

Radioligand binding

Human cloned α_1-AR subtypes: Competition curves were determined in triplicate, in membranes from rat-1 cells expressing individual α_1-AR subtypes with a final [^{125}I]-iodo-2[β-(4-hydroxyphenyl)-ethylaminomethyl]-tetralone ([^{125}I]-HEAT) concentration of 100 pM, as previously described [14]. Curves were fitted and *pKi* (affinity constant*:* -log of *Ki*) calculated by using non-linear regression analysis (GraphPad Software, San Diego, CA, USA) from the displacement curves obtained in each case with 8 – 9 different concentrations of the tested compound.

Rat cerebral cortical membranes: Membranes were prepared from cerebral cortex of female Wistar rats as reported elsewhere [9]. Competition curves were determined in triplicate, with a final [^3H]-prazosin or [^3H]-(+)-*cis*-diltiazem concentration of 0.2 nM or 3 nM, as previously described [6], [9]. Curves were fitted and *pKi* calculated by using non-linear regression analysis (GraphPad Software, San Diego, CA, USA) from the displacement curves obtained in each case with 10 – 12 different concentrations of the tested compound.

Isolation and determination of PDE isoform activity

Cytosolic PDE (PDE1, PDE3, PDE4 and PDE5) were isolated from the media layer of bovine aorta by a modification of the method of Lugnier et al. [15]. Cytosolic PDE2 was isolated from cultured bovine aortic endothelial cells by HPLC chromatography [16]. PDE activities were measured as previously described [6], [7], [15], at a substrate ([^3H]-cyclic AMP or [^3H]-cyclic GMP) concentration of 1 μM. The concentration needed to produce 50% inhibition (IC_{50}) was determined by non-linear regression analysis (Graph Pad Software, San Diego, CA, USA.) from the concentration-response curves obtained in each case with 6 different concentrations of the tested compound.

Functional studies in isolated organ bath

These studies were carried out according to the general procedure described by Gisbert et al. [17]. Concentration-response relaxation curves were obtained by addition of cumulative concentrations of the compounds to aortic or tail artery rings denuded of endothelium, in which maximal sustained contractions had been induced by NA (1 μM or 10 μM, respectively) in Ca^{2+}-containing solution. Concentration-response relaxation curves were also performed on aortic rings previously contracted with KCl (80 mM) plus phentolamine (10 μM). Relaxations are expressed as percentages of the maximum increment in tension obtained by agonist addition. The effect of different concentrations of each alkaloid added 15 min before was tested on NA-induced contraction in Ca^2-free medium, following the general procedure described by Gisbert et al. [17]. The concentration needed to produce 50% relaxation or inhibition (IC_{50}) was obtained from a non-

linear regression analysis (Graph Pad Software, San Diego, CA, USA) from the displacement curves obtained in each case with 6 (Ca^2-free medium) or 9 (Ca^{2+}-containing solution) different concentrations of the tested compound.

Accumulation of [^3H]-inositol phosphates

The method used to determine the accumulation of inositol phosphates was described previously [17]. Cerebral cortex slices (350 μm cubes) and tail artery rings (5 mm) were labelled with 10 μCi ml^{-1}*myo*-[^3H]-inositol. To determine possible agonist or antagonist actions of the alkaloids, the tissues (50 μL of packed cortex slices or two tail artery rings) were incubated for 30 min in the absence (basal) or presence of 100 μM of the different alkaloids, or the samples were stimulated for 30 min with a maximal concentration of NA (100 μM in cortex and 10 μM in tail artery) in the absence or presence of the alkaloid (100 μM). Each sample was performed in triplicate. Accumulation of [^3H]-inositol phosphates was calculated as a percentage (dpm%) of total [^3H]-inositol labelled lipids in each individual sample to correct for inter-experimental variations in labelling and sample sizes, and was expressed as percentages of the unstimulated [^3H]-inositol phosphate accumulation (basal).

Data analysis

The results are presented as the mean ± SEM or 95% confidence intervals for *n* determinations obtained from different animals or cell preparations. Where ANOVA showed significant differences ($P < 0.05$), the results were further analyzed using the Student-Newman-Keuls test and differences were considered significant when $P < 0.05$.

Drugs, radioisotopes, chemicals and solutions

The following drugs were used: the alkaloids *(R)*(−)-anonaine {(purity > 95%; $[\alpha]_D$: −55° (*c* 0.08; EtOH)} and *(R)*(−)-roemerine {(purity > 95%; $[\alpha]_D$: −56° (*c* 0.06; EtOH)} were isolated from *Annona cherimolia* [8]; *(R)*(−)-pukateine was isolated from *Laurelia novae-zelandiae* bark as described elsewhere [18] {the purified sample has m. p. 216 – 217 °C; $[\alpha]_D$: −251° (*c* 0.8; EtOH), appeared to be homogeneous on TLC, and exhibited no peaks attributable to impurities in its 300 MHz ^{1}H-NMR spectrum}; acetylcholine chloride, *cis*-(+)-diltiazem hydrochloride, (−)-NA bitartrate, prazosin hydrochloride were purchased from Sigma (St. Louis MO, USA) and phentolamine mesylate was purchased from RBI (Natick MA, USA).

The radioisotopes used were (sources in parentheses): [7-*methoxy*-^3H]-prazosin (72 – 78 Ci mmol^{-1}), *myo*-[^3H]-inositol with PT6 – 271 stabilizer (80 – 110 Ci mmol^{-1}) (Amersham International, Buckinghamshire, UK); [^{125}I]-HEAT (2200 Ci mmol^{-1}), *cis-(+)*-[*N*-methyl-^3H]-diltiazem (80 – 85 Ci mmol^{-1}) (DuPont-New England Nuclear, Boston; MA); [8 – ^3H]-guanosine 3′,5′-cyclic phosphate ammonium salt (5 – 15 Ci mmol^{-1}), [2,8 – ^3H]-adenosine 3′,5′-cyclic phosphate ammonium salt (30 – 50 Ci mmol^{-1}) (New England Nuclear, UK). All other reagents were of analytical grade.

The appropriate dilutions of the following drugs were prepared daily before the experiments from concentrated stock solutions (10^{-2} M) kept at −20 °C: acetylcholine (to test the presence or absence of the endothelium in functional studies in isolated organ bath), phentolamine or diltiazem (to determine non-specific [^3H]-prazosin or [^3H]-diltiazem binding, respectively). Alkaloid solutions were prepared daily in deionized water with 0.1% (w/v) ascorbic acid. In all tests carried out in this work, deionized water and/or the appropriate dilutions of ascorbic acid had no significant pharmacological effects.

Results

Ligand binding assays

Human cloned α_1-AR subtypes: The three human cloned α_1-AR subtypes demonstrated high and approximately equal affinity for [^{125}I]-HEAT (pK_D = 9.88, 9.92 and 9.89 for α_{1A}-, α_{1B}- and α_{1D}-subtypes, respectively) [14]. All compounds tested competed for [^{125}I]-HEAT binding in membranes from rat-1 cells stably expressing cloned α_1-AR subtypes with steep and monophasic curves. Prazosin showed the same affinity for the three AR subtypes but the alkaloids tested exhibited significantly higher affinity for α_{1A}- and α_{1D}- than for α_{1B}-AR. Anonaine and roemerine were significantly more potent for the α_{1A}- than for the α_{1D}-subtype, the affinity order being $\alpha_{1A} > \alpha_{1D} > \alpha_{1B}$. Pukateine was slightly more potent for the α_{1D} subtype, but this difference was not significant, the affinity order being $\alpha_{1A} = \alpha_{1D} > \alpha_{1B}$ (Table **1**). Comparing the results obtained with the different alkaloids, methylation of the nitrogen atom (roemerine *versus* anonaine) significantly increases the affinity for all three α_1-AR subtypes (Table **1**). The introduction of a hydroxy group at C-11 (pukateine *versus* roemerine) significantly decreases the affinity for α_{1A}- and α_{1B}-AR without changing the affinity for the α_{1D}-subtype (Table **1**).

Table **1** Affinity (*pKi*) for the aporphine alkaloids at human cloned α_1-AR subtypes as determined by competition experiments

Drug	α_{1A}	α_{1B}	α_{1D}	*Selectivity Order*
Prazosin[a]	9.90 ± 0.16	9.50 ± 0.15	10.4 ± 0.09	$\alpha_{1A} = \alpha_{1B} = \alpha_{1D}$
(*R*)(−)-Anonaine	6.19 ± 0.12*	5.13 ± 0.10*	5.64 ± 0.13*	$\alpha_{1A}^{\circ\circ\circ\cdot} > \alpha_{1D}^{\circ\circ} > \alpha_{1B}$
(*R*)(−)-Roemerine	6.61 ± 0.12	5.53 ± 0.04	6.22 ± 0.12	$\alpha_{1A}^{\circ\circ\circ\cdot} > \alpha_{1D}^{\circ\circ\circ} > \alpha_{1B}$
(*R*)(−)-Pukateine	5.84 ± 0.13***	5.00 ± 0.09**	6.10 ± 0.07	$\alpha_{1A}^{\circ\circ\circ} = \alpha_{1D}^{\circ\circ\circ} > \alpha_{1B}$

Data are expressed as mean values of the tested agents ± SEM.
n = 3 – 6 individual experiments for each compound performed in triplicate.
* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ *versus* (*R*)(−)-roemerine.
°° $P < 0.01$, and °°° $P < 0.001$ *versus* α_{1B}.
· $P < 0.05$ *versus* α_{1D}.
[a] According to data previously published [14].

Rat cerebral cortical membranes: [^3H]-Prazosin and [^3H]-(+)-*cis*-diltiazem bound to rat cerebral cortex with a dissociation constant pK_D of 9.85 and 7.30, respectively [6]. The specific binding of 0.2 nM [^3H]-prazosin was completely inhibited by the alkaloids, roemerine being the most potent and pukateine the least potent inhibitor (Table **2**).

These alkaloids also inhibited the binding of 3 nM [^3H]-(+)-*cis*-diltiazem, but with significantly ($P < 0.001$) lower affinity than that shown for [^3H]-prazosin binding (Table **2**). Methylation of the nitrogen atom (roemerine *versus* anonaine) significantly increased affinity for the benzothiazepine binding site at the Ca^{2+} channel, whereas the introduction of a hydroxy group at C-11 (pukateine *versus* roemerine) led to a significant decrease (Table **2**).

Prazosin inhibited [^3H]-prazosin binding without affecting [^3H]-(+)-*cis*-diltiazem binding at a concentration of 0.1 μM (Table **2**).

Effect on bovine aorta PDE

We examined the inhibitory effects of the alkaloids on the different cytosolic molecular forms of PDE isolated from bovine aortic smooth muscle (PDE1, PDE3, PDE4 and PDE5) [6], [19] and from bovine aortic endothelial cells (PDE 2) [16]. Bovine, human and rat aorta show the same families of PDE, and similar results in biochemical studies are obtained in these tissues [15]. As shown in Table **3**, whereas papaverine inhibited all PDE activities in a non-specific manner [6], none of the methylenedioxyaporphines had a significant effect as inhibitor of the different PDE forms assayed, the IC_{50} being bigger than 100 μM in all cases.

Functional studies in isolated organ bath

The magnitude of the sustained contractile response induced by NA (1 or 10 μM) in rat aorta or tail artery rings denuded of endothelium and incubated in Ca^{2+}-containing solution was 720 ± 61 mg (n = 10) and 674.3 ± 60.6 mg (n = 10), respectively. These concentrations of NA have been shown to elicit maximal contractions in each tissue. The contractile response of rat aorta elicited by depolarizing solution (KCl 80 mM) in the presence of phentolamine (10 μM) was 590.8 ± 43.1 mg (n = 12).

Addition of cumulative concentration of the alkaloids (0.1 – 100 μM) induced concentration-dependent relaxation in aorta and tail artery contracted with NA in Ca^{2+}-containing solution. The pIC_{50} values obtained are shown in Table **4**. The relative order of potency was different in tail artery than in aorta. Roemerine was significantly more potent than anonaine both in aorta and tail arteries, whereas pukateine exhibited significantly lower potency than roemerine only in rat tail artery.

These compounds have also a relaxant effect on KCl-depolarized rat aorta but with a lower potency than that shown against NA-induced contraction, pukateine being the less active compound (Table **4**).

To exclude the possible activity of the alkaloid as blockers of Ca^{2+} channels we also tested the effects of these compounds on NA-induced contractions in rat aorta in Ca^{2+}-free medium. After 20 min in Ca^{2+}-free solution, addition of NA (1 μM) induced a phasic contraction (190.4 ± 22.1 mg, n = 10), followed by a tonic one (108.8 ± 10.7 mg, n = 10). After a loading period (20 min) in Krebs to refill intracellular Ca^{2+} stores, a second addition of NA in Ca^{2+}-free solution induced a similar response in control experiments but a weaker response or none at all in the presence of different concentrations of the test compounds (0.1 – 100μM). The pIC_{50} values obtained with the different alkaloids were similar to those obtained in the presence of Ca^{2+} (Table **4**). The relaxant potency showed by prazosin on NA-induced contraction in rat aorta was also similar in the presence or absence of Ca^{2+}.

Table **2** Affinity (*pKi*) for the aporphine alkaloids *versus* [^3H]-prazosin binding or [^3H]-(+)-*cis*-diltiazem binding to specific receptors of rat cerebral cortex membranes determined by competition experiments

	[^{3}H]-prazosin	*[^{3}H]-(+)-cis-diltiazem*
Prazosin	9.75 ± 0.08	N.I.
(*R*)(–)-Anonaine	5.91 ± 0.05$^{**\circ\circ\circ}$	4.99 ± 0.05*
(*R*)(–)-Roemerine	6.18 ± 0.04$^{\circ\circ\circ}$	5.26 ± 0.08
(*R*)(–)-Pukateine	5.72 ± 0.07$^{***\circ\circ\circ}$	4.62 ± 0.10**

Data are expressed as mean values of the tested agents ± SEM.
n = 4 – 9 individual experiments for each compound performed in triplicate.
* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ *versus* (*R*)(–)-roemerine.
$^{\circ\circ\circ}$ $P < 0.001$ *versus* [^3H]-(+)-*cis*-diltiazem.
N.I.: no inhibition at 0.1 μM.

Table **3** Effects of aporphine alkaloids on PDE isolated from bovine aorta

	PDE1		*PDE2*		*PDE3*	*PDE4*	*PDE5*
	-CaM	*+CaM*	*-cGMP*	*+cGMP*			
Papaverine[a]	5.10	4.56	N.D.	N.D.	6.12	5.77	5.97
(*R*)(–)-Anonaine	(26%)	3.74 ± 0.05	(41%)	3.55 ± 0.05	3.36 ± 0.03	3.69 ± 0.03	3.76 ± 0.04
(*R*)(–)-Roemerine	(37%)	3.90 ± 0.03	3.35 ± 0.03	3.64 ± 0.04	3.16 ± 0.04	3.87 ± 0.03	3.59 ± 0.05
(*R*)(–)-Pukateine	3.40 ± 0.02	3.48 ± 0.04	(39%)	3.28 ± 0.05	(38%)	3.56 ± 0.04	3.86 ± 0.03

pIC_{50} are expressed as mean values of the tested agents ± SEM from two determinations obtained on different enzymatic preparations performed in duplicate. Numbers in parentheses indicate % inhibition of the enzyme at 1 mM drug concentration. N.D.: Not determined.
PDE activity was assessed with 1 μM [^3H]-cyclic GMP + 1 mM EGTA or 1 μM [^3H]-cyclic GMP + 10 μM $CaCl_2$ + 18 nM calmodulin (CaM) for PDE1; 1 μM [^3H]-cyclic AMP + 1 mM EGTA with or without 5 μM cyclic GMP for PDE2; 1 μM [^3H]-cyclic AMP + 1 mM EGTA and in the presence of 50 μM rolipram for PDE3 or 100 μM cyclic GMP for PDE4 in order to limit the cross-contamination of these forms; 1 μM [^3H]-cyclic GMP + 1 mM EGTA for PDE5.
[a] Data from Ivorra et al. [6].

Table **4** Inhibitory potencies (pIC_{50}) of alkaloids on contractions induced by NA in Ca^{2+}-containing solution [NA-Ca^{2+}(+)] or in Ca^{2+}-free solution [NA-Ca^{2+}(–)] in rat aorta (NA 1 μM) and in rat tail artery (NA 10 μM) or by KCl (80 mM) plus phentolamine (10 μM) in rat aorta

	Aorta			*Tail Artery*
	NA-Ca^{2+}(+)	***NA-Ca^{2+}(–)***	***KCl***	***NA-Ca^{2+}(+)***
Prazosin[a]	9.68 ± 0.15 *n* = 9	9.45 (9.24 – 9.66) *n* = 4 – 5	4.42 ± 0.19 *n* = 4	8.46 ± 0.07 *n* = 8
(*R*)(–)-Anonaine	5.12 ± 0.09* *n* = 10	4.94 (4.90 – 4.97) *n* = 3 – 5	4.76 ± 0.08° *n* = 5	5.44 ± 0.08 **° *n* = 10
(*R*)(–)-Roemerine	5.42 ± 0.06 *n* = 9	5.22 (5.08 – 5.36) *n* = 3 – 5	4.80 ± 0.06°°° *n* = 5	5.81 ± 0.05°°° *n* = 9
(*R*)(–)-Pukateine	5.19 ± 0.10 *n* = 10	4.99 (4.92 – 5.07) *n* = 3 – 5	4.27 ± 0.03***°°° *n* = 7	5.55 ± 0.08 *° *n* = 8

Data are expressed as mean values of the tested agents ± SEM, except the values of pIC_{50} on NA-Ca^{2+}(–), which are presented as mean with 95% confidence intervals.
n = number of experiments.
* $P < 0.05$, ** $P < 0.01$ *versus* (*R*)(–)-roemerine.
° $P < 0.05$, °°°$P < 0.001$ *versus* aorta NA-Ca^{2+} (+).
[a] Data from Gisbert et al. [17] and Ivorra et al. [6].
The experiments were performed in the absence of endothelium.

Effect of the alkaloids on phosphoinositide metabolism

In rat tail artery and cerebral cortex, NA (10 μM or 100 μM, respectively) increased [^3H]-inositol phosphates accumulation (70 ± 3 dpm%, n = 4 or 21 ± 2 dpm%, n = 6, respectively) compared to unstimulated basal values (9 ± 1 dpm%, n = 4 or 10 ± 1 dpm%, n = 6, respectively). These concentrations of NA have been shown to induce maximal response in each tissue.

Prazosin significantly inhibited the increase in [^3H]-inositol phosphate formation induced by NA in rat tail artery, and cerebral cortex corroborating that this increase is related to α_1-AR stimulation. Anonaine, roemerine and pukateine also inhibited the accumulation of [^3H]-inositol phosphates induced by NA in both tissues. All three alkaloids (100 μM) showed significantly stronger inhibition ($p < 0.01$), in rat tail artery than in rat cerebral cortex (Fig. **2**). The effects of these compounds on inositol phosphate formation were also assayed in both tissues, and it was found that they did not increase the inositol phosphate levels above the basal values (results not shown).

Discussion

All three *(R)*-1,2-methylenedioxyaporphines assayed showed affinity for the three human cloned α_1-AR subtypes, and exhibited slightly but significantly lower affinities for the α_{1B}- with regard to the α_{1A}- or α_{1D}-subtypes. Affinities for the different subtypes were in the order $\alpha_{1A} > \alpha_{1D} > \alpha_{1B}$ for anonaine and roemerine, and $\alpha_{1A} = \alpha_{1D} > \alpha_{1B}$ for pukateine. Thus, the (*R*)-1,2-methylenedioxyaporphines, like the other sets of aporphines tested previously [9], [10], [11], [12], show different affinities for α_1-AR subtypes.

Comparing the alkaloids among themselves we can see that the presence of a hydroxy group at C-11 (pukateine *versus* roemerine) is associated with a significant decrease in affinity for the α_{1A}- and α_{1B}-subtypes without changing the affinity for the α_{1D}-AR. Earlier results obtained in our laboratory had indicated the importance of certain hydroxy substituents on one or the other of the aporphine aromatic rings as determinants of the affinity of these alkaloids for each subtype. In a series of 1,2,9,10-tetraoxygenated *(S)*-aporphine alkaloids a free hydroxy group at C-2 is

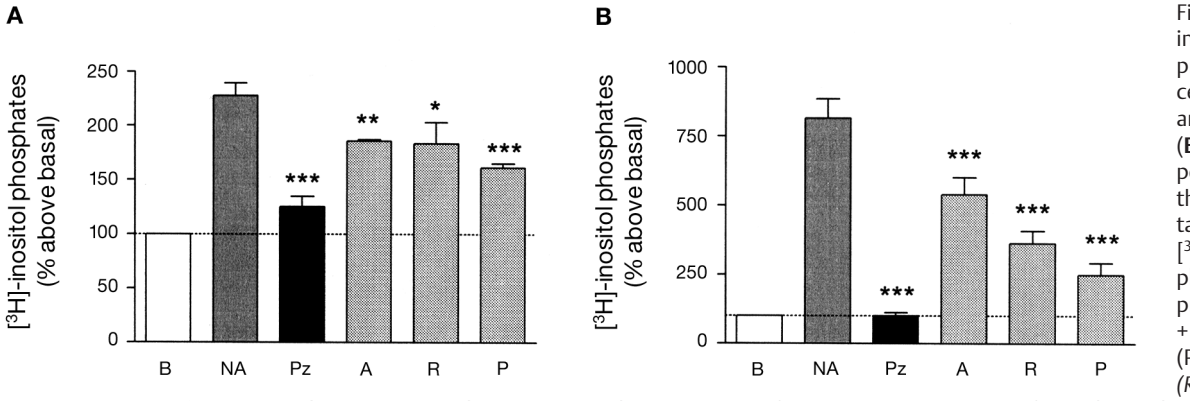


Fig. **2** Total [^3H]-inositol phosphate production in rat cerebral cortex (**A**) and in rat tail artery (**B**) expressed as percentage above the basal value (**B**) taken as 100%. [^3H]-Inositol phosphate levels in the presence of NA, NA + 1 μM prazosin (Pz), NA + 100 μM *(R)*(–)-anonaine (**A**), *(R)*(–)-roemerine (R) or *(R)*(–)-pukateine (P). Results are expressed as mean ± SEM from 3 – 4 experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *versus* [^3H]-inositol phosphate accumulation induced by NA.

associated with increased affinity for α_{1A}-AR, and to a lesser extent for the α_{1B}-subtype, while it does not affect the affinity of the alkaloid for α_{1D}-AR [9], [10], [11]. Conversely, in a set of (*R*)-10,11-dihydroxyaporphines, a free hydroxy group at C-10 increases the affinity for α_{1D}-AR without changing the affinity for the α_{1A}- or α_{1B}- subtypes [12]. In conclusion, the present results confirm and extend the view that the α_1-AR subtype selectivity of aporphine alkaloids can be modulated by the position of free hydroxy substituents on the aromatic rings of the aporphine system, suggesting that the α_1-AR recognition sites for the aromatic rings of the aporphine structure are different in each of the three subtypes.

On the other hand, introduction of a methyl group on the nitrogen atom (roemerine *versus* anonaine) significantly increases affinity for all three subtypes. We have previously reported similar results for another set of aporphine alkaloids [12]. The fact that *N*-methylation leads to increased affinity for the three α_1-AR subtypes supports the hypothesis that the topography of the region of the binding site recognizing the basic or protonated nitrogen atom is similar in all three receptor subtypes.

Other authors working with other aporphine alkaloids [2], [3] have also evidenced the importance of free hydroxy groups and the *N*-methyl substitution on the aporphine skeleton on α_1-AR activity.

Binding studies on native AR present in rat cerebral cortex, which has been reported to possess a mixed population of α_{1A}- and α_{1B}-AR [9], confirms the results obtained in cloned α_1-AR subtypes. The order of affinities obtained in brain tissue follows the same pattern as that observed in human cloned α_{1A}- and α_{1B}-AR, and structural changes give identical changes in affinity: *N*-methylation increases affinity and C-11 hydroxylation decreases affinity for [^{3}*H*]-prazosin binding sites in rat cerebral cortex.

Our previous studies had also shown that some aporphine structures act not only at α_1-AR, but at higher concentrations also possess Ca^{2+} entry blocking properties mediated by their binding at the benzothiazepine sites [6], [7], [8]. In order to ascertain the selectivity of methylenedioxyaporphines for these two mechanisms of action, we have also tested the effects of these compounds on [^{3}*H*]-diltiazem binding in rat cerebral cortical membranes and on KCl-depolarized rat aorta. All three alkaloids now tested showed greater affinity as inhibitors of [^{3}*H*]-prazosin than of [^{3}*H*]-(+)-*cis*-diltiazem binding, indicating that the introduction of a 1,2-methylenedioxy group in the aporphine skeleton does not modify the selectivity of action for α_1-AR previously reported for aporphine structures [6], [7], [8]. The relaxation induced by these alkaloids on KCl-induced contractions in rat aorta, suggests a Ca^{2+}-channel blocker activity for these compounds. The potency of these compounds as Ca^{2+}-channel blockers was lower than that shown as α_1-AR antagonists.

Analyzing the structural features, we can see that the presence of a methyl group on the nitrogen atom (roemerine *versus* anonaine) was associated with increased affinity as a Ca^{2+} channel blocker, while the presence of a hydroxy group at C-11 (pukateine) led to decreased affinity. Similar effects of hydroxy substitution at other positions of the aporphine structure have been reported previously [6], [7], suggesting that the presence of polar hydroxy groups may impede the interaction between the compound and the benzothiazepine site of the Ca^{2+} channel. Taken together, our results allow us to conclude that *N*-methylation favours the interaction of aporphines with α_1-AR and voltage-gated Ca^{2+} channels. However, the presence of hydroxy groups on the aporphine aromatic rings has different effects on the affinity of these alkaloids for α_1-AR subtypes but always decreases the affinity for Ca^{2+}-channels.

Another possible mode of action of these compounds is as inhibitors of PDE, since papaverine, a tetramethoxybenzylisoquinoline alkaloid structurally related to aporphines, is a non-selective PDE inhibitor [6]. Our previous results have shown that aporphines generally have low potency as PDE inhibitors and, among the structures tested, only 6a(*S*)-(+)-glaucine (1,2,9,10-tetramethoxyaporphine) exhibited activity inhibiting a single form of this enzyme (PDE4) [6]. However, at that time the effect of methylenedioxyaporphines was not investigated. The results obtained in the present work show that none of the methylenedioxy alkaloids tested was able to inhibit significantly any of the different vascular smooth muscle PDE or PDE2 isolated from vascular endothelial cells, ruling out a possible activity of these alkaloids at this level.

Finally, we studied the functional activity of these natural products as vasorelaxants by testing their effects on NA-induced contractions in two isolated vascular smooth muscle preparations, rat aorta and rat tail artery, with different functional populations of α_1-AR subtypes (α_{1A} in rat tail artery and α_{1D} in rat aorta [17]). All three alkaloids acted as antagonists inhibiting NA-induced contraction in the two vascular preparations. These compounds exhibited a similar effect on NA-induced contraction in Ca^{2+}-containing and Ca^{2+}-free solution in rat aorta. These results, together with the lack of inhibitory activity on PDE, relate the vasorelaxant effect of these compounds to an antagonist action on α_1-AR. This hypothesis is corroborated by the fact that all these compounds were able to inhibit the NA-induced formation of [^{3}*H*]-inositol phosphates in different rat tissues like tail artery and cerebral cortex.

Moreover, the results obtained in functional assays in tail artery (α_{1A}) and aorta (α_{1D}) show the same selectivity profile observed in binding experiments performed in human cloned α_1-AR subtypes, for the structural changes that modify the affinity for α_{1A} or α_{1D} cloned AR also modify the activity on tail artery or rat aorta, respectively: *N*-methylation increases the activity both in tail artery and aorta, whereas hydroxy substitution decreases it significantly only in tail artery. Although functional studies are lacking for many aporphines at α_1-AR subtypes, it is interesting to note that these compounds seem to be antagonists regardless of their configuration at C-6a. In particular, the antagonist activity of pukateine now demonstrated by us contrasts with its agonist activity at striatal dopamine receptors [20], which it seems to share with other C-11-hydroxylated (*R*)-aporphines [21].

In conclusion, the present investigation has demonstrated that the three *(R)*-1,2-methylendioxyaporphines now tested by us act as vasorelaxants mainly because of α_1-AR antagonist activity. If we compare the compounds, we observe that *N*-methylation

improves the interaction of the compounds with all α_1-AR subtypes, whereas the insertion of a hydroxy group at C-11 decreases the affinity for α_{1A}- and α_{1B}-AR subtype and does not change the affinity for α_{1D}-AR. On the basis of these results and our earlier ones it may be stated that the position of hydroxy groups on the aromatic rings of the aporphine structure plays an important role in determining the selectivity for the different α_1-AR subtypes. The use of these sterically constrained alkaloids may helpful in searching for clues about the binding site topography at the different α_1-AR subtypes. Moreover, these natural plant alkaloids may be useful as a structural model for the design and subsequent development of new and more selective α_1-blocking drugs.

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