Evidence for the involvement of imidazoline receptors in the central hypotensive effect of rilmenidine in the rabbit

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1 Rilmenidine has recently been introduced as a new centrally-acting antihypertensive agent. We examined its cardiovascular effects after intracerebral injection to anaesthetized rabbits. Cumulative doses of rilmenidine injected intracisternally (1 to 300 $\mu\text{g kg}^{-1}$) led to dose-dependent decreases in arterial blood pressure and heart rate. The effective doses of rilmenidine were lower when injected centrally than when injected intravenously.

2 Pretreatment with the same dose of yohimbine or idazoxan shifted the rilmenidine dose-response curves for its hypotensive and bradycardic effects to the right. Idazoxan, which has an imidazoline structure, proved to be a more active antagonist than yohimbine of rilmenidine centrally-mediated cardiovascular effects.

3 The dose-response curve for the central hypotensive effect of rilmenidine was also shifted to the right after pretreatment with a bovine brain extract. This extract contains the endogenous ligand of the imidazoline-preferring receptors which is not a catecholamine.

4 Rilmenidine, like clonidine, proved to be active when micro-injected into the rabbit nucleus reticularis lateralis region.

5 In conclusion, rilmenidine exhibited in the rabbit a central hypotensive effect which originated in the same area as where clonidine acts. Specific imidazoline-preferring receptors appear to be involved in this hypotensive effect.

Introduction

Rilmenidine is an oxazoline recently developed as a clinical antihypertensive drug (Fillastre *et al.*, 1988). Experiments performed in dogs, cats and rats have suggested the existence of a central component in its hypotensive effect (Laubie *et al.*, 1985; Van Zwieten *et al.*, 1986; Koenig Bérard *et al.*, 1988). In addition, studies performed in both animals and man, have shown that hypotensive doses of the drug do not evoke any significant sedative effect (Fillastre *et al.*, 1988).

Thus the pharmacological profile underlying the hypotensive effect of this drug seemed an interesting study. The aim of the present work was to confirm the central hypotensive effect of rilmenidine and to investigate its mechanism of action in a species suitable for the study of this type of central hypotensive drug, i.e. the rabbit. Therefore, using various techniques, we injected rilmenidine directly into rabbits' brains and recorded cardiovascular parameters in order to determine its site of action. These experiments were performed with reference to previous studies used to locate the site of action of clonidine and clonidine-like molecules (Bousquet *et al.*, 1981; 1984; Wolf & Mohrland, 1984; Head & De Jong, 1986; Ernsberger *et al.*, 1987; Granata & Reis, 1987; McAuley *et al.*, 1989). Experiments using antagonists were carried out to provide some information as to the possible mechanism of action.

Several years ago we demonstrated that, in the cat, the major site for the central hypotensive effect of clonidine was within the nucleus reticularis lateralis (NRL) area of the medulla (Bousquet & Guertzenstein, 1973; Bousquet *et al.*, 1981). More recently, a structure-activity study proved that the imidazolines were able to induce such effects although catecholamines or phenylethylamines could not (Bousquet *et al.*, 1984). These results allowed us to propose the existence of imidazoline-sensitive, catecholamine-insensitive receptors involved both in the hypotensive effect of the imidazolines and in the central regulation of arterial blood pressure (for review see Bousquet *et al.*, 1989). These receptors would seem to be located mainly within the NRL region and imidazoline preferring receptors have recently been characterized in this region

both in bovine and human brain (Ernsberger *et al.*, 1987; Bricca *et al.*, 1988; 1989a). An endogenous ligand for this receptor has been partially purified (Atlas & Burnstein, 1984a,b; Meeley *et al.*, 1986; Ernsberger *et al.*, 1988). In fact, a partially purified brain extract contains a substance which is not a catecholamine but displaces [³H]-clonidine bound to brain membrane preparations and increases blood pressure whereas clonidine itself reduces it (Atlas & Burnstein, 1984a,b; Bousquet *et al.*, 1986). This substance has been provisionally called 'clonidine displacing substance' (CDS). In addition, we recently observed, through binding tests on human NRL membrane preparations (Bricca *et al.*, 1989b) that rilmenidine, structurally very close to the imidazoline, exhibited a relative selectivity for these receptors. The selectivity of rilmenidine was about 2.5 times higher than that of clonidine for the medullary imidazoline receptors as compared to the cortical α_2 -adrenoceptors (Bricca *et al.*, 1989b). In the present work we have investigated further the central site of action as well as the hypotensive mechanism of action of rilmenidine in the rabbit.

Methods

General procedure

Normotensive male rabbits (Fauves de Bourgogne) anaesthetized with pentobarbitone (30 mg kg^{-1}) injected through the marginal vein of the ear were tracheotomized, immobilized with pancuronium bromide (2 mg kg^{-1} , i.v.) and artificially ventilated (Bird Mark 8). Arterial blood pressure was recorded with the aid of a catheter placed within the abdominal aorta by way of the right femoral artery. This catheter was connected to a pressure transducer (Statham P23Db) which in turn was connected to a Gould recorder. Instantaneous systolic and diastolic arterial pressures were recorded and mean arterial pressure calculated. Heart rate was calculated from the blood pressure signal.

Intracerebral injections

The animal's head was placed in a stereotaxic frame (La Précision Cinématographique, Française). Drugs were either

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injected intracisternally (100 μ l) directly within the cisterna magna using cumulative doses, or micro-injected with one dose per animal into the NRL area (1 μ l). In order to inject drugs directly into the NRL region, we placed unilaterally within the brain tissue a needle with an outer diameter of 100 μ m. To expose the dorsal surface of the medulla oblongata, the neck muscles were separated from the occipital protuberance and reclined downward. After removing the atlanto-occipital membrane, the occipital bone was resected. By means of a micromanipulator, the needle was positioned at an angle of 30° to the horizontal, 2 mm laterally from the median line and 4 mm above the obex. Then it was inserted for 7.5–8 mm into the medulla oblongata.

At the end of each experiment, Evans blue was injected under the same conditions as the drugs. After the animals were killed, the brain was removed, the brainstem dissected and fixed in 10% formalin. Subsequent histological controls on 50 μ m frozen sections confirmed the location of the needle tip and diffusion of the solution in the NRL area, i.e. close to the ventral surface of the medulla oblongata within a region located between the trapezoid bodies rostrally and the pyramids laterally.

At the beginning of each experiment, prior to any drug injection, equal volumes of vehicle were given. Sometimes transient cardiovascular changes were observed after the stereotaxic insertion of the needle within the medulla oblongata, in which case the drug micro-injections were only performed after these parameters had returned to their initial values. Vehicle never produced any significant effect on haemodynamic parameters. In addition, the effects of the drugs were measured only when a steady-state effect was observed, i.e. 5 to 10 min after central or systemic injections. In antagonist experiments, yohimbine, idazoxan or CDS were injected as pretreatment in one dose, 10 min before the injection of cumulative doses of rilmenidine.

Brain extract preparation

Fresh bovine brains were obtained from the local slaughterhouse and rapidly brought, on ice, to the laboratory where dissection took place within one hour.

The chromatographic beds were purchased from Bio-Rad (U.S.A.), the chemicals for Fluka (Switzerland) and the 3 H-labelled molecules from New England Nuclear.

The extraction procedure was based upon the protocol described previously by Atlas & Burstein (1984a,b) with some modifications. Instead of being heated, the 50 000 g supernatant was ultrafiltered through a 1000 daltons cut-off membrane. The resulting dialysate was lyophilised, extracted twice with methanol and the last extract evaporated to dryness under vacuum at 40°C.

The crude extract was submitted to gel filtration chromatography on a BioGel P2 column (length = 100 cm, diameter = 2.6 cm) eluted with a 0.1 M NH_4HCO_3 , pH 7.9 buffer at 5°C (flow rate = 30 ml h⁻¹) and monitored at 206 nm. Ion-exchange chromatography using CM-cellulose was performed on the active fraction. The column (length = 10 cm, diameter = 1.6 cm, flow rate = 20 ml h⁻¹) was step eluted with a 0.01 M HCOONH_4 , pH 4.0 buffer followed by 3 ml of 1 M NH_4OH . After each step, the biological activities of each fraction were tested. The binding assays were performed according to the techniques described elsewhere (Atlas *et al.*, 1987; Bricca *et al.*, 1989a). At each step the fraction selected was the one which preferentially displaced [3 H]-clonidine from its binding to human NRL membranes as compared with [3 H]-rauwolscine binding to rat cortex membranes, representing the α_2 -adrenoceptor model. That extract was named endazoline.

Endazoline was dissolved in 0.9% w/v NaCl solution (saline). One unit of endazoline is defined as the amount needed to displace 50% of [3 H]-clonidine specifically bound to human NRL membranes.

Drugs

The following drugs were used: rilmenidine (S3341, Institut de Recherches Servier), clonidine (Sigma), pentobarbitone Na (Nembutal, Abbott), pancuronium bromide (Pavulon, Organon), yohimbine (Sigma) and ($\pm$)-idazoxan (RX 781094, Institut de Recherches Servier). Drugs were dissolved in saline. The pH of every solution was adjusted to 7.5.

Statistics and calculations

Data in the text are given as means $\pm$ s.e.mean. For each group, the log dose-response curves were used to determine the dose which decreased mean blood pressure by 20% of the initial value (D_{20}). We compared the effects of rilmenidine without and after treatment with the antagonists. Variance analysis of the basal values were performed in order to test the homogeneity of the mean arterial pressure in the different groups. To test the effect of the drug alone, comparisons were made by Student's paired *t* test. Statistical analyses were performed using two-way analyses of variance for repeated measures. The remaining data were compared by Newman-Keuls's test. *P* values < 0.05 were used as the criterion of significance and *n* is the number of experiments. The calculations were made by computer assisted analyses with STAT-ITCF programme (I.T.C.F. Boigneville-Maisse, France).

Results

Controls

The five groups of animals were highly homogeneous regarding their average preinjection values of blood pressure. Analyses of variance showed no significant difference in the basal values of the mean arterial pressure (MAP) ($F = 2.14$; $P > 0.05$) and heart rate (HR) ($F = 1.30$; $P > 0.05$) between all the groups of animals.

Cardiovascular effects of intravenous injection of rilmenidine

Cumulative doses of rilmenidine (1 μ g to 1 mg kg⁻¹) were injected intravenously to 5 normotensive anaesthetized rabbits. Under such conditions, a dose-dependent decrease of the MAP was observed. The MAP decreased from 92 ± 5 mmHg to 52 ± 5 mmHg ($P < 0.001$) at the maximal dose of 1 mg kg⁻¹. The HR also decreased from 266 ± 12 beats min⁻¹ to 188 ± 6 beats min⁻¹ ($P < 0.001$) (Figure 1). Thus rilmenidine injected intravenously induced a hypotensive effect which became significant at a dose of 30 μ g kg⁻¹. This effect was associated with bradycardia. The dose which

Figure 1 Changes in mean arterial pressure (MAP, ●) and heart rate (HR, ■) with cumulative doses of rilmenidine administered intravenously into pentobarbitone-anaesthetized rabbits ($n = 5$). Vertical bars show s.e.mean.

decreased MAP by 20% (D_{20}) was $70 \mu\text{g kg}^{-1}$ in this series. At this dose, the HR decreased by 16%.

Cardiovascular effects of intracisternal injection of rilmenidine

Cumulative doses of rilmenidine (1 to $300 \mu\text{g kg}^{-1}$) were injected to 7 anaesthetized rabbits. A dose-dependent decrease of MAP was observed. At the maximal dose of $300 \mu\text{g kg}^{-1}$, MAP fell from $99 \pm 3 \text{ mmHg}$ to $59 \pm 2 \text{ mmHg}$ ($P < 0.001$). At this dose the heart rate also decreased from $276 \pm 10 \text{ beats min}^{-1}$ to $190 \pm 7 \text{ beats min}^{-1}$ ($P < 0.001$) (Figure 2). The D_{20} for fall in MAP was $1.5 \mu\text{g kg}^{-1}$ in this series of experiments at which dose the heart rate decreased less than 10%.

Antagonism experiments

Pretreatment with yohimbine Low doses of yohimbine ($1.5 \mu\text{g kg}^{-1}$ i.e. 5 nmol kg^{-1}) were given intracisternally to a series of 5 anaesthetized rabbits prior to the injection of cumulative doses of rilmenidine (1 to $300 \mu\text{g kg}^{-1}$). Yohimbine itself

did not significantly affect the cardiovascular parameters. The pre-rilmenidine value of MAP was $100 \pm 2 \text{ mmHg}$ in this series of experiments. Yohimbine provoked a moderate shift to the right of the dose-response curve obtained with cumulative doses of intracisternal rilmenidine, the D_{20} of rilmenidine being approximately $30 \mu\text{g kg}^{-1}$. The bradycardic effect of rilmenidine was not significantly affected by yohimbine (Figure 2).

Pretreatment with idazoxan ($\pm$)Idazoxan was given intracisternally ($1 \mu\text{g kg}^{-1}$ i.e. 5 nmol kg^{-1}) to 5 anaesthetized rabbits prior to rilmenidine injection. Idazoxan itself never influenced the cardiovascular parameters. The dose-effect curves for rilmenidine were displaced toward the right by this pretreatment. At the highest dose of rilmenidine ($300 \mu\text{g kg}^{-1}$), MAP decreased from $95 \pm 5 \text{ mmHg}$ to $71 \pm 9 \text{ mmHg}$ ($P < 0.05$) and the heart rate was reduced from $283 \pm 14 \text{ beats min}^{-1}$ to $227 \pm 14 \text{ beats min}^{-1}$ ($P < 0.05$). After pretreatment with idazoxan, the rilmenidine D_{20} was around $180 \mu\text{g kg}^{-1}$ for MAP (Figure 2).

Pretreatment with endazoline When given intracisternally to 5 anaesthetized rabbits (500 units in $100 \mu\text{l}$), endazoline caused a transient and variable increase in blood pressure. The average values of MAP before endazoline administration were not significantly different ($112 \pm 6.5 \text{ mmHg}$) 10 min after endazoline pretreatment ($116 \pm 10 \text{ mmHg}$) ($P > 0.05$). The dose-response curve for rilmenidine was shifted to the right. At the maximal dose of $300 \mu\text{g kg}^{-1}$ of rilmenidine, MAP decreased from $116 \pm 10 \text{ mmHg}$ to $84 \pm 6 \text{ mmHg}$ ($P < 0.001$). Heart rate decreased from $240 \pm 11 \text{ beats min}^{-1}$ to $174 \pm 14 \text{ beats min}^{-1}$ ($P < 0.05$). The rilmenidine D_{20} was around $30 \mu\text{g kg}^{-1}$ for MAP and HR after endazoline treatment (Figure 2).

A two way analysis of variance using Newman-Keuls's test showed that pretreatment with the antagonists significantly affected ($P < 0.05$) the effects of rilmenidine on MAP ($F = 25.34$) and HR ($F = 15.08$).

Effects of injection of rilmenidine and clonidine into the NRL

Rilmenidine effects Five anaesthetized rabbits were injected with $2 \mu\text{g kg}^{-1}$ rilmenidine into the NRL. MAP decreased from $99 \pm 4 \text{ mmHg}$ to $75 \pm 7 \text{ mmHg}$ ($P < 0.001$) (i.e. $-24 \pm 2\%$) and the heart rate was decreased by $12 \pm 3\%$ ($266 \pm 16 \text{ beats min}^{-1}$ to $232 \pm 6 \text{ beats min}^{-1}$) ($P < 0.05$) (Figure 3).

Effects of clonidine injection into the rabbit's NRL Under the same experimental conditions, 5 anaesthetized rabbits were injected with $0.06 \mu\text{g kg}^{-1}$ clonidine directly into the NRL. A significant drop of $26 \pm 2\%$ in the MAP, from $87 \pm 6 \text{ mmHg}$ to $72 \pm 5 \text{ mmHg}$ ($P < 0.001$), was observed. The heart rate

Figure 2 Changes in mean arterial pressure (MAP, a) and heart rate (HR, b) with cumulative doses of rilmenidine injected intracisternally (i. cist) into anaesthetized rabbits: (◆) control ($n = 5$); (▲) rilmenidine ($n = 5$); (▼) pretreatment with yohimbine i. cist. ($n = 5$); (●) pretreatment with idazoxan i. cist. ($n = 5$); (■) pretreatment with endazoline i. cist. ($n = 5$). Vertical bars show s.e.mean.

Figure 3 Effects on arterial pressure (AP) of a unilateral microinjection of rilmenidine and clonidine into the nucleus reticularis late-ralis of the anaesthetized rabbit.

also decreased from 242 ± 16 beats min^{-1} to 190 ± 15 beats min^{-1} , i.e. $11 \pm 4\%$ ($P < 0.05$) (Figure 3).

Discussion

In the present study, we compared the cardiovascular effects of cumulative doses of rilmenidine given systemically or intracisternally to anaesthetized rabbits. The intracisternal injection allowed the substance to reach the medullary region very quickly. In fact, the most probable location of the site for the central hypotensive action of rilmenidine was the NRL area of the brainstem, by analogy with the site of action for clonidine (Bousquet *et al.*, 1981; 1984).

We have demonstrated that when injected intracisternally, 40 times lower doses of rilmenidine are able to lower arterial blood pressure than when injected systemically, indeed the D_{20} ratio was of the order of 45.

Rilmenidine proved capable of lowering MAP by about 40% when centrally injected. As this substance was active when injected into the brain at doses which had no effect when given systemically, one can assume the existence of a central hypotensive action of rilmenidine in the rabbit. This observation confirms the results obtained by other authors in cats or dogs (Laubie *et al.*, 1985; Van Zwieten *et al.*, 1986).

It was of importance to locate precisely the site of action of the substance within the medulla oblongata and it was necessary to verify whether this site was the same as that involved in the effects of clonidine. As it has never been proved that clonidine has an effect in the rabbit within the same area as in the cat, namely the NRL, we checked this point first. As we have shown significant decreases in rabbit MAP after micro-injections of very low doses of clonidine (60 ng kg^{-1}), we can reasonably conclude that in the rabbit at least, a main site for the central hypotensive action of this drug is located within the NRL region. In addition, we have shown that doses of rilmenidine, as low as $2 \mu\text{g kg}^{-1}$, when micro-injected in the same area are also able to decrease significantly arterial blood pressure. The blood pressure lowering effect of rilmenidine was always accompanied by a bradycardia.

Van Zwieten *et al.* (1986) reported that yohimbine ($30 \mu\text{g kg}^{-1}$), a well-known antagonist of the cardiovascular effects of clonidine totally prevented, when injected into the cat's vertebral artery, the hypotensive effects of a standard dose of rilmenidine. Recently, we studied the ability of very low doses of yohimbine to antagonize the central cardiovascular effects of clonidine in the rat and observed that pretreatment with doses as low as $1 \mu\text{g kg}^{-1}$, were able to antagonize the hypotensive effects of clonidine in the SHR (Tibiriça *et al.*, 1988).

If we regard the numerous studies evaluating α -adrenoceptor antagonist potencies of various substances in classical pharmacological tests, it appears from results obtained with catecholamines particularly noradrenaline as agonists, that idazoxan and yohimbine have very similar potencies. In contrast, when imidazolines were used as agonists in the same tests, the results are very similar to those obtained in the present work. Indeed idazoxan proved to be more potent than yohimbine for example, as an antagonist of various effects of clonidine (Chapleo *et al.*, 1981; Doxey *et al.*,

1983; 1984). Thus we have now investigated the effects of yohimbine and idazoxan, given as pretreatment at the same low doses in rabbits, towards the central hypotensive effect of rilmenidine. Yohimbine, at 5 nmol kg^{-1} given intracisternally, did not affect resting haemodynamic parameters but partially prevented the blood pressure decrease induced by rilmenidine. Indeed the dose-response curve obtained after intracisternal injection of cumulative doses of rilmenidine was shifted to the right. The D_{20} ratio was around 20. However, when the same dose of idazoxan was given intracisternally, a more marked and significant shift of the dose-effect curve of rilmenidine was observed (D_{20} ratio = 120). This suggests that idazoxan behaves as a more potent antagonist of the hypotensive effects of rilmenidine than yohimbine. Our hypothesis to explain the higher antagonist activity of idazoxan as compared to yohimbine is that the chemical structure (imidazoline) confers to the former a better affinity and selectivity for the imidazoline receptors. Recent binding studies showing that yohimbine cannot compete with [^3H]-idazoxan for specific imidazoline binding sites corroborate this hypothesis (Hamilton *et al.*, 1988). The results observed in antagonist experiments are consistent with the view that antagonists with imidazoline structure better prevent the rilmenidine central hypotensive effects than an antagonist with a non-imidazolinic structure, such as yohimbine.

The endogenous ligand for the imidazoline-preferring receptors contained within the bovine brain extracts which we prepared according to a method derived from that of Atlas & Burstein (1984a,b) is a substance corresponding to the following criteria: (1) it preferentially competes with clonidine for the imidazoline preferring binding sites; (2) it has proven hypertensive effects whereas clonidine exhibits a hypotensive effect; (3) it is not a catecholamine.

We propose to name this extract 'endazoline' in order to show that it corresponds to more restrictive and thus more precise criteria than those applied to the former CDS, which was defined only by clonidine binding displacement in rat cortical membrane preparations which contain mainly α_2 -adrenoceptors.

In the present work, we observed that pretreatment with endazoline also shifted to the right the dose-effect curves of the central hypotensive action of rilmenidine. These data confirm that the hypotensive effect of rilmenidine originating in the NRL region may involve imidazoline-preferring receptors.

In conclusion, we have shown the existence of a central hypotensive effect of rilmenidine associated with bradycardia in the rabbit. At least in part, the origin of the rilmenidine central hypotensive effect was located within the NRL area, which is the region where clonidine induces its effect. The cardiovascular effect of rilmenidine was well antagonized by a drug with an imidazoline structure: idazoxan. Our results suggest that the hypotensive effect of rilmenidine might involve imidazoline-specific receptors, thus corroborating the rilmenidine selectivity for these receptors already suggested by binding tests (Bricca *et al.*, 1989b).

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