ELSEVIER

European Journal of Pharmacology 311 (1996) 277–283

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Coexpression of α_{1A}- and α_{1B}-adrenoceptors in the liver of the rhesus monkey (*Macaca mulatta*)

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Received 25 March 1996; revised 23 May 1996; accepted 29 May 1996

Abstract

The α_1-adrenoceptors present in the liver of rhesus monkeys was characterized using [^{3}H]prazosin. This radioligand binds to monkey liver membranes with high affinity (K_D 0.33 nM) to a moderately abundant number of sites (97 fmol/mg of protein). These sites were characterized pharmacologically, by binding competition, observing two affinities for most ligands. The order of potency for agonists was: (a) for the high affinity sites: oximetazoline > epinephrine = norepinephrine > methoxamine; and (b) for the other sites (low affinity for the α_{1A}-adrenoceptor-selective agonists): oximetazoline ≥ epinephrine = norepinephrine ≫ methoxamine. For antagonists the orders of potency were: (a) for the high affinity sites: *R*-(−)-5[2-[[2-(ethoxyphenoxy)ethyl]amino]propyl]-2-metoxybenzenesulfonamide HCl (tamsulosin) ≥ 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane (WB4101) ≥ prazosin ≥ (+)- niguldipine > 5-methyl-urapidil = benoxathian > phentolamine > 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride (BMY 7378); (b) for the other sites (low affinity for the α_{1A}-adrenoceptor-selective antagonists): prazosin > tamsulosin > phentolamine = WB4101 > (+)-niguldipine ≥ 5-methyl-urapidil = benoxathian > BMY 7378. These data strongly suggest that *Macaca mulatta* liver cells coexpress α_{1A}- and α_{1B}-adrenoceptors. Expression of the mRNA for these receptors was confirmed by reverse transcriptase-polymerase chain reactions.

Keywords: α_1-Adrenoceptor; Liver; Hepatocyte; (Rhesus monkey)

1. Introduction

α_1-Adrenoceptors constitute an heterogeneous family of receptors; three subtypes have been cloned (Cotecchia et al., 1988; Schwinn et al., 1990; Lomasney et al., 1991; Perez et al., 1991). As recommended by the IUPHAR most recent consensus update (Hieble et al., 1995), the cloned receptors (lower case) are referred to as α_{1a}-, α_{1b}- and α_{1d}-adrenoceptors which correspond to the pharmacologically defined (upper case) α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors.

Catecholamines are among the main modulators of liver metabolism (Hems and Whitton, 1980), both α_1- and β_2-adrenoceptors seem to mediate such modulation. The relative roles of these adrenoceptors and their densities in hepatocytes vary considerably among species (Sulakhe et al., 1988). It has been previously observed that there is considerable variation in the subtype of α_1-adrenoceptor expressed in the liver of animals of different species. Thus, rats (Han et al., 1987; García-Sáinz et al., 1992a, 1994), hamsters (García-Sáinz et al., 1994), mice (García-Sáinz et al., 1994), chickens (Gutiérrez-Venegas and García-Sáinz, 1993) and fish (*Ichtalurus punctatus*) (García-Sáinz et al., 1995a) express the α_{1B} subtype, whereas rabbits (García-Sáinz et al., 1992a, 1995d; Taddei et al., 1993), guinea pigs (García-Sáinz et al., 1992a,b, 1995d), dogs (García-Sáinz et al., 1995b) and humans (García-Sáinz et al., 1995c) express predominantly the α_{1A} subtype in their livers.

Rhesus liver is a very frequently used model to study drug metabolism (Marselos and Tomatis, 1992), toxicology (Marselos and Tomatis, 1992; Thorgeirsson et al., 1994), carcinogenesis (Marselos and Tomatis, 1992; Thorgeirsson et al., 1994), and viral (Persidsky et al., 1994) and parasitic (Yang et al., 1994) infections. Surprisingly and, to the best of our knowledge, the adrenoceptors present in the liver of rhesus monkeys have not been characterized. The α_1-adrenoceptors present in the liver of rhesus monkey was investigated and the results are here

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PII S0014-2999(96)00433-5

presented. Our data indicate that rhesus liver coexpresses α_{1A}- and α_{1B}-adrenoceptors.

2. Materials and methods

(−)-Epinephrine, (−)-norepinephrine, oxymetazoline, prazosin and guanylyl-imido-diphosphate (Gpp(NH)p) were obtained from Sigma Chemical Co. Benoxathian, 5-methyl-urapidil, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride (BMY 7378) and 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane (WB4101) were from Research Biochemicals. Phentolamine (Ciba-Geigy), methoxamine (Burroughs Wellcome), *R*-(−)-5[2-[[2-(ethoxyphenoxy)ethyl]amino]propyl]-2-metoxybenzenesulfonamide HCl (tamsulosin) (Yamanouchi Europe) and (+)-niguldipine (Byk Gulden) were generous gifts from the indicated pharmaceutical companies. [^{3}H]Prazosin (71.8 Ci/mmol) was from New England Nuclear. [α-^{32}P]dCTP (6000 Ci/mmol) and the Nick translation kit were obtained from Amersham. Restriction enzymes were obtained from New England BioLabs.

Rhesus monkeys (pathogen-free, obtained from Charles) were maintained and killed according to the principles and guidelines of our Institute and the NIH, to avoid any unnecessary pain to the animals. The monkeys were used for electrophysiological studies (Ruiz et al., 1995) in the laboratory of Dr Ranulfo Romo, who kindly provided the livers of the animals to us, immediately after the animals were subjected to general anesthesia and sacrificed. Four animals with the following characteristics were used: (1) male, 5.5 kg, ≈ 3 years old; (2) female, 6.5 kg, ≈ 4.5 years old; (3) male, 3.5 kg, ≈ 3 years old; (4) male, 18 kg, ≈ 6.5 years old. Binding studies were performed with membranes from all the animals; no difference was observed among them and the data were pooled. Partially purified liver plasma membranes, were obtained by the method of Neville (1968). Membranes were washed and resuspended in 50 mM Tris, 10 mM $MgCl_2$, pH 7.5; aliquots of the membrane preparation were frozen and stored under liquid nitrogen until used (usually within two weeks), without any noticeable change in receptor binding. Radioligand binding studies were performed by incubating membranes (100–150 μg protein) in 50 mM Tris, 10 mM $MgCl_2$, pH 7.5 with the radioactive ligand, alone or with the indicated agents. Incubations were in a total volume of 0.25 ml for 60 min in a water bath shaker at 25°C. At the end of the incubation, 2.5 ml of ice-cold buffer were added to the membrane suspension, the membranes were immediately filtered on GF/C filters and washed three times (2.5 ml each time) with the same buffer. Saturation experiments were performed using concentrations between 0.05–12 nM [^{3}H]prazosin and binding competition studies with 1–1.5 nM of the radioactive ligand (75–85% receptor occupation). Non-specific binding was evaluated in the presence of 10 μM phentolamine; specific binding represented 70–80% of the total binding at the K_D. Binding competition experiments with agonists were performed in the presence of 100 μM Gpp(NH)p. When the sensitivity to inactivation by chloroethylclonidine was tested, liver membranes were preincubated with different concentrations of this irreversible antagonist, washed and the specific binding at ≈ 80% saturation was determined. Binding saturation and competition data were analyzed using the EBDA and LIGAND (Munson and Rodbard, 1980) programs (Elsevier-Biosoft). Two-state fits were utilized only when this, more complex model, significantly improved the goodness of the fit. K_i values were calculated according to Cheng and Prusoff (1973). Protein was quantified by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Total RNA was obtained by the method of Chomczynski and Sacchi (1987) with minor modifications. RNA integrity was routinely checked by electrophoresis on formaldehyde-containing agarose gels and visualization under UV light using ethidium bromide staining. Reverse transcriptase-polymerase chain reactions (RT-PCR) were performed with total RNA using a kit from Perkin Elmer. The primers: 5′-CATCGTGGTCGGCTGCTTCGTC-CTCTGCTG-3′ (coding sense) and 5′-TCCCACGGG-GATGCGCACCATGTCCTTGTG-3′ (anticoding sense) corresponding to bases 1261–1290 and 1547–1576 of the human α_{1a}-adrenoceptor cDNA (Hirasawa et al., 1993), were employed: the PCR amplification profile consisted of denaturation at 94°C for 1 min, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min (40 cycles) using 5 μg of total RNA. The primers 5′-GCTCCTTCTACATC-CCGCTCG-3′ (coding sense) and 5′-AGAACACCACCT-TGAACACGG-3′ (anticoding sense), corresponding to bases 629–649 and 983–1003 of the rat α_{1b}-adrenoceptor cDNA (Voigt et al., 1990) were employed with the following PCR conditions: denaturation 95°C for 1 min, primer annealing at 58.5°C for 1 min, and extension at 72°C for 1 min (40 cycles) using 5 μg of total RNA. RT-PCR mixtures were heated initially at 95°C for 3 min and at the end of the reaction cycles were incubated at 72°C for 7 min. The RT-PCR products were electrophoresed in 7.5% polyacrylamide gels, transferred to nylon membranes and hybridized under high stringency conditions (García-Sáinz et al., 1992a) with the whole bovine cDNA α_{1a}-adrenoceptor (Schwinn et al., 1990) or the whole hamster (DDT_1 MF-2 cells) α_{1b}-adrenoceptor cDNA (Cotecchia et al., 1988) labeled by Nick Translation (specific activity ≈ 10^8 cpm/μg).

3. Results

[^{3}H]Prazosin binding to rhesus monkey liver plasma membranes was rapid (reaching steady state in ≈ 5–7 min, data not shown) and saturable. A representative bind-

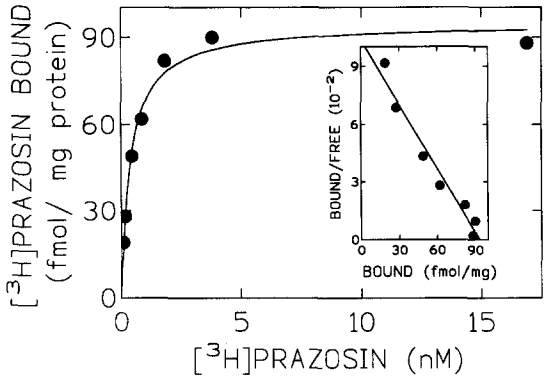

Fig. 1. [^{3}H]Prazosin saturation isothern using rhesus monkey liver membranes. Plotted are the specific bindings and the Rosenthal analysis (inset). These data are representative of 3 separate experiments with each of the 4 different membrane preparations.

ing saturation isotherm is presented in Fig. 1. It can be observed that [^{3}H]prazosin binds with high affinity (K_D 0.33 ± 0.03 nM) to a moderately abundant number of sites (97 ± 12 fmol/mg of protein) (results are the means ± S.E.M of 3 separate experiments performed with each of the four different membrane preparations). The Rosenthal transformation (Fig. 1, inset) of the data yielded a straight line, suggesting that [^{3}H]prazosin binds to sites with homogeneous affinity for this radioligand.

Chloroethylclonidine is an alkylating antagonist that is able to irreversible inactivate some α_1-adrenoceptors (Minneman, 1988). As it is shown in Fig. 2, preincubation with chloroethylclonidine dose dependently inactivated a large proportion of the [^{3}H]prazosin binding sites present in these membranes.

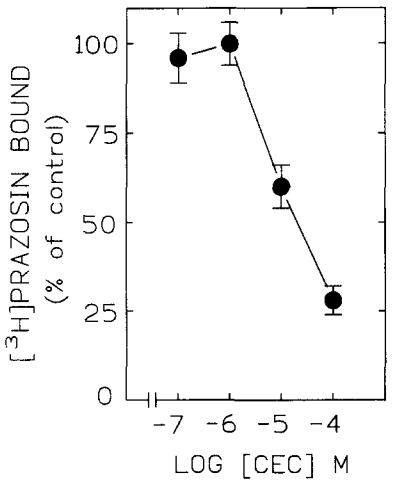

Fig. 2. Effect of chloroethylclonidine on [^{3}H]prazosin specific binding. Membranes were preincubated with the indicated concentration of clorethylclonidine (CEC), washed and the specific [^{3}H]prazosin binding determined. Results are presented as percentage of the specific binding observed in membranes preincubated without chloroethylclonidine (control). Plotted are the means and vertical lines represent the S.E.M. of 2 separate experiments using each of the 4 different membrane preparations.

We further characterized the [^{3}H]prazosin binding sites present in these membranes by binding competition studies using selective agonists and antagonists. In preliminary experiments, it was observed that the slope of the competition for agonists was consistently much below 1, suggesting heterogeneity. Such heterogeneity could be due to the presence of several receptor subtypes, to the existence of more than one affinity state for agonists for a given receptor subtype, or to both, i.e., more than one receptor subtype each with more than one state of affinity for agonists. The last possibility proved to be correct. Studies with antagonists indicated receptor heterogeneity (i.e., the presence of more than one receptor subtype in the membrane preparations, see below). In addition, we observed that the hydrolysis-resistant analogue of GTP, Gpp(NH)p, shifted to the right and increased the slope, of the binding competition curve for epinephrine; the binding competition curve for an antagonist, such as phentolamine, was not modified by the GTP analogue (data not shown). These data suggested that guanine nucleotide-modulated interconversion of two affinity states for agonists was taking place, in agreement with the ternary complex model (De Lean et al., 1980; Samama et al., 1993). Therefore, in order to better characterize the α_1-adrenoceptor subtypes present in rhesus monkey liver membranes we performed all binding competition experiments with agonists in the presence of 100 μM Gpp(NH)p.

Binding competition experiments using agonists are presented in Fig. 3 and Table 1. It can be observed that the order of potency for the agonists was: oximetazoline > epinephrine ≥ norepinephrine > methoxamine. Interestingly, in spite of the presence of Gpp(NH)p in the binding assays, the competition curves for oximetazoline and methoxamine were clearly biphasic and best fitted to a two-site fit (Fig. 3). The K_H and K_L values and the proportion of site with each affinity are presented in Table

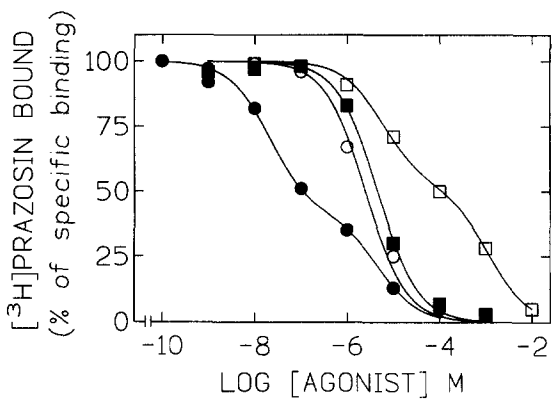

Fig. 3. Binding competition experiments with agonists using rhesus monkey liver membranes. The following agonists were employed: oxymetazoline (filled circles), epinephrine (open circles), norepinephrine (filled squares) and methoxamine (open squares). The figure is representative of 4 separate experiments using each of the 4 different membrane preparations.

Table 1
Binding parameters derived from the competition by agonists and antagonists for [^{3}H]prazosin binding sites in rhesus monkey liver membranes

	K_H (nM)	K_L (nM)	R_H (%)	R_L (%)
Agonists				
Oximetazoline	6.38±1.25	395±144	41±5	58±5
(−)-Epinephrine	533±69	–	100	–
(−)-Norepinephrine	830±210	–	100	–
Methoxamine	1957±502	265000±26495	48±4	51±4
Antagonists				
Prazosin	0.26±0.03	–	100	–
Tamsulosin	0.16±0.05	3.60±0.85	57±7	42±7
(+)-Niguldipine	0.64±0.34	149±52	50±5	50±5
WB4101	0.23±0.04	20±5	52±6	48±6
5-Methyl-urapidil	2.60±0.05	210±37	55±5	45±5
Benoxathian	3.00±0.95	228±33	63±5	36±5
Phentolamine	19±2	–	100	–
BMY 7378	326±42	–	100	–

Results are the means±S.E.M. of 4–6 determinations using 4 different membrane preparations.

1. Binding competition curves with the natural catecholamines, epinephrine and norepinephrine, were consistently monophasic (Fig. 3 and Table 1).

In the binding competition studies using antagonists, we observed that only the curves for prazosin, phentolamine and BMY 7378 were monophasic and best fitted to a one-state fit (Fig. 4 and Table 1). In contrast, the competition curves for the other antagonists tested (tamsulosin, (+)-niguldipine, WB4101, 5-methyl-urapidil and benoxathian) were consistently biphasic and best fitted to two-state fits (Fig. 4 and Table 1). When two different affinities for an agent were detected, the proportion was ≈ 1 : 1.

Using RT-PCR we detected a fragment of 316 bp using

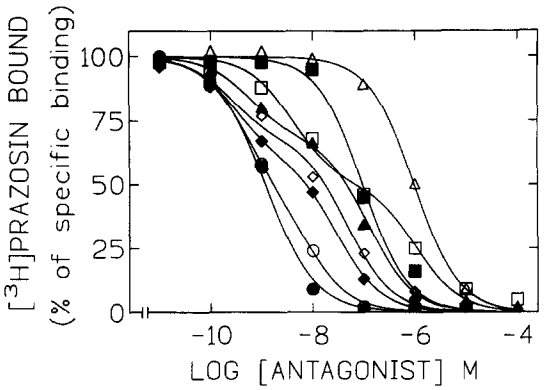

Fig. 4. Binding competition experiments with antagonists using rhesus monkey liver membranes. The following antagonists were employed: prazosin (filled circles), tamsulosin (open circles), (+)-niguldipine (filled diamonds), WB4101 (open diamonds), 5-methyl-urapidil (filled triangles), benoxathian (open squares), phentolamine (filled squares) and BMY 7378 (open tringles). The figure is representative of 4 separate experiments using each of the 4 different membrane preparations.

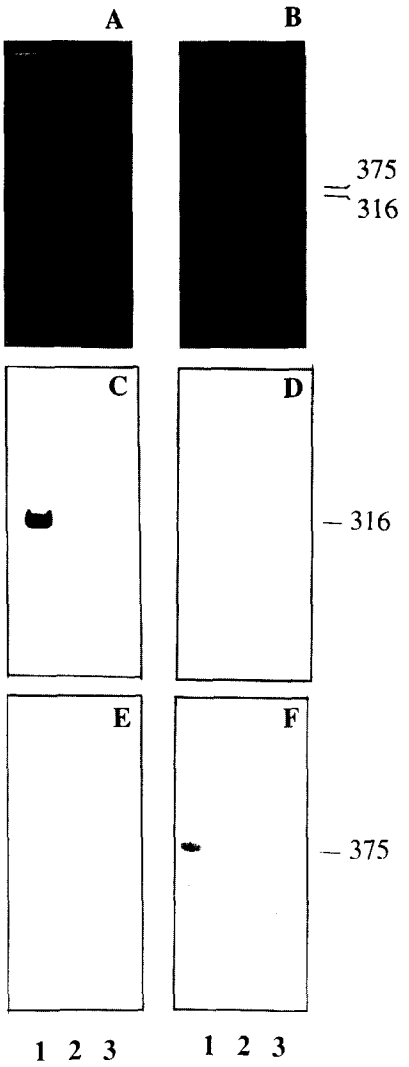

Fig. 5. RT-PCR and hybridization of the reaction products. Ethidium bromide-stained gels showing the RT-PCR products obtained using conditions for the α_{1A} subtype (panel A) and for the α_{1B} subtype (panel B) (lanes 1, complete reaction; lanes 2, reaction performed in the absence of RNA; lanes 3, reaction performed in the absence of reverse transcriptase). Gels identical to those in panels A and B were transferred to nylon membranes and hybridized with the labeled α_{1a} cDNA (panels C and D) or with the labeled α_{1b} cDNA (panels E and F); autoradiographs presented are representative of 3 different experiments with identical results.

the primers and conditions for the α_{1A}-adrenoceptor and another fragment of 375 bp using the primers and conditions for the α_{1B}-adrenoceptor (Fig. 5, panels A and B). No such fragments were detected in the absence of RNA or reverse transcriptase (Fig. 5, panels A and B). The fragments had the sizes expected (comparable to those obtained using RNA from cells transfected with the cloned receptor subtypes (data not shown)). The identity of the fragments was further tested by hybridization with the whole bovine α_{1a}-adrenoceptor cDNA (Fig. 5, panels C

and D) and with the whole hamster α_{1b}-adrenoceptor cDNA (Fig. 5, panels E and F).

4. Discussion

In the present study we characterized the α-adrenoceptors of rhesus monkey liver. The characterization was performed using complementary approaches, radioligand binding and mRNA expression using RT-PCR. The results of both approaches were consistent and clearly indicate that in the liver of *Macaca mulatta*, two adrenoceptor subtypes, i.e., the α_{1A}- and the α_{1B}-adrenoceptors, are expressed.

Our laboratory has characterized the α_1-adrenoceptors present in the liver of different species. The number of sites detected in the liver of animals of different species varies markedly. We have observed values as high as 500–900 fmol/mg of membrane protein in the livers of rats, mice or hamsters (García-Sáinz et al., 1994) and as low as 30 fmol/mg membrane protein in fish liver (García-Sáinz et al., 1995a). Although differences in the recovery of plasma membrane protein during the isolation procedures exist, they do not seem to explain the enormous variations in receptor densities that we have observed. In this regard, the density of sites detected in rhesus monkey liver can be considered as moderately abundant.

The pharmacological characteristics of the [^{3}H]prazosin binding sites, detected in the present study, strongly suggest coexpression of α_{1A}- and α_{1B}-adrenoceptors. For antagonists: tamsulosin (Michel and Insel, 1994; García-Sáinz et al., 1995d), (+)-niguldipine (Boer et al., 1988), WB4101 (Morrow and Creese, 1986), 5-methyl-urapidil (Gross et al., 1988) and benoxathian (Michel et al., 1989) have considerable bigger affinity for α_{1A}-adrenoceptors than for the other subtypes whereas prazosin is considered non selective for the different subtypes (Bylund et al., 1994). BMY 7378 is a selective antagonists ($K_i \approx 2$ nM) for the α_{1D} subtype (Goetz et al., 1995; Piascik et al., 1995). For the agonists: oximetazoline and methoxamine have also bigger affinity for the α_{1A} subtype (Bylund et al., 1994). The order of potency for agonists for both binding sites was similar, i.e., (a) for the high affinity sites: oximetazoline > epinephrine = norepinephrine > methoxamine; and (b) for the other sites (low affinity for the α_{1A}-adrenoceptor-selective agonists): oximetazoline ≥ epinephrine = norepinephrine ≫ methoxamine. For antagonists the orders of potency were as follows: (a) for the high affinity sites: tamsulosin ≥ WB4101 ≥ prazosin ≥ (+)-niguldipine > 5-methyl-urapidil = benoxathian > phentolamine > BMY 7378; (b) for the other sites (low affinity for the α_{1A}-adrenoceptor-selective antagonists): prazosin > tamsulosin > phentolamine = WB4101 > (+)-niguldipine ≥ 5-methyl-urapidil = benoxathian > BMY 7378. The orders of potency and the K_i values are consistent with the classification of these sites as representing α_{1A}- and α_{1B}-adrenoceptors, respectively. The monophasic competition curve and the very low affinity for BMY 7378 strongly suggests that α_{1D}-adrenoceptors are not present in rhesus liver membranes. It is important to mention that, as expected, the same ratio (≈ 1 : 1) was consistently observed with all the agents that show subtype selectivity.

The binding results were confirmed by the molecular biological approach. We attempted to detect the expression of the receptor subtype mRNAs through Northern analysis, but the level of expression was apparently rather low, and the amount of signal was very small. Nevertheless, using RT-PCR the data clearly showed the expression of selective mRNA for both the α_{1A} and the α_{1B} subtypes.

Our data clearly indicated that the liver from the rhesus monkey coexpresses α_{1A}- and α_{1B}-adrenoceptors. α_{1A}-Adrenoceptors have been detected in the liver of rabbits (García-Sáinz et al., 1992a, 1995d; Taddei et al., 1993), guinea pigs (García-Sáinz et al., 1992a,b, 1995d), dogs (García-Sáinz et al., 1995b) and humans (García-Sáinz et al., 1995c), but not in the liver of animals of other species, more commonly used in the laboratory, such as rats (Han et al., 1987; García-Sáinz et al., 1992a, 1994), hamsters (García-Sáinz et al., 1994) or mice (García-Sáinz et al., 1994) which express the α_{1B} subtype. At this point, it is not possible to define why some species express in their livers α_{1A}- or α_{1B}-adrenoceptors or both subtypes. The α_{1B} subtype is expressed in the liver of lower vertebrates (such as fishes) whereas the α_{1A} subtype is observed in the liver of higher vertebrates. This suggests that the former was probably expressed in this organ, earlier during evolution. It is interesting that the adrenergic innervation of mammalian liver seems to differ among species (Moghimzadeh et al., 1983) and that such innervation can be clearly detected in the livers of humans, guinea pigs, rabbits and also in the rhesus monkey, all of which express the α_{1A} subtype, but that no such parenchymal innervation can be detected in the liver of rats or mice (which express the α_{1B} subtype). Correlation between innervation and expression of a given subtype seems to exist but, at this point, there is no evidence for a causal relationship.

Detection of a receptor does not indicate the function that such receptor may have in an organ and even less if such receptor is relevant in a physiological context. As mentioned before, catecholamines are among the main modulators of liver metabolism (Hems and Whitton, 1980) in most species and α_1-adrenoceptors play a cardinal role. In addition, there is evidence that α_1-adrenoceptors are implicated in the control of hepatocyte proliferation (Cruise et al., 1987) and changes in the expression levels of this receptor family have been observed in regenerating and neoplastic hepatic tissue (Cruise et al., 1987; Kost et al., 1992). The physiological role(s) that these receptors play in the rhesus monkey remain to be determined. Finally, it is worth mentioning that the liver is a complex organ in which several cell types coexist and interact in a paracrine fashion. Therefore, the type of cells in which these recep-

tor subtypes are located within the whole organ seems also to be of major importance. The methodology employed, in this study, for membrane purification, predominantly isolates basolateral membranes from parenchymal cells. However, even among parenchymal cells, there is heterogeneity in function. Certainly, further experiments will be required to address all these aspects.

Acknowledgements

The authors express their gratitude to Dr Ranulfo Romo for generously providing the liver of the animals used in this work. This research was partially supported by Grants from CONACyT (4711-N9406) and DGAPA (IN 200193).

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