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BINDING OF CATECHOLAMINES TO *ALPHA*-1 ACID GLYCOPROTEIN, ALBUMIN AND LIPOPROTEINS IN HUMAN SERUM

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Abstract—The binding of catecholamines in human serum was determined by equilibrium dialysis at 37°. For serum concentrations of 10–15 nM the bound fractions were 28.8 ± 2.2%, 25.7 ± 1.7% and 22.2 ± 2.2% for (±)-isoproterenol (IPR), (±)-norepinephrine (NE) and (±)-epinephrine (EPI), respectively. At higher serum concentrations saturation occurred. *Alpha*-1 acid glycoprotein (AAG) possessed one high affinity binding site and approximately 10 low affinity sites. The catecholamines were bound to AAG with the same order of potency for both classes of binding sites: IPR (K_{d_1}: 100 μM K_{d_2}: 2.2 mM) > NE (K_{d_1}: 120 μM, K_{d_2}: 6.5 mM) > EPI (K_{d_1}: 140 μM, K_{d_2}: 14 mM). Human serum albumin (HSA) and lipoproteins (SLP) interacted with the catecholamines in a non-saturable manner. IPR showed the strongest and EPI the weakest association to both of these serum protein fractions. (−)-Propranolol was able to inhibit the binding of IPR in serum and to isolated AAG, but not to HSA or to SLP. The present results show that AAG is an important catecholamine-binding protein in human serum. AAG, but not HSA or SLP, possesses binding sites shared by adrenergic receptor stimulators and blockers.

Previous studies have reported that albumin [1–3] and various globulins [3, 4] are responsible for the binding of catecholamines in human serum/plasma [3, 5, 6]. The observation that IPR, NE and EPI‡ were able to displace high affinity bound propranolol in serum [7], coupled to the report that the high affinity propranolol binding site in serum [8] and the propranolol binding site on isolated AAG [9, 10] have identical dissociation constants, suggests that catecholamines also interact with AAG in human serum. The present work was performed firstly to determine whether AAG is a binding protein for catecholamines in human serum and thereafter to characterize further the binding of catecholamines to AAG, HSA and SLP and in full serum.

MATERIALS AND METHODS

Chemicals. ^{3}H-(±)-Isoproterenol hydrochloride (spec. act. 12.2 Ci/mmol) and ^{3}H-(±)-epinephrine hydrochloride (spec. act. 11.3 Ci/mmol) were purchased from Amersham Int. plc. (Bucks, U.K.). ^{3}H-(±)-Norepinephrine hydrochloride (spec. act. 15.4 Ci/mmol) and ^{3}H-(−)-propranolol hydrochloride (spec. act. 19.6 Ci/mmol) were purchased from New England Nuclear (Dreieich, F.R.G.). The purity of the radiolabelled ligands was checked by thin layer chromatography in one of the recommended solvent systems prior to use. The following unlabelled compounds were employed: (±)-isoproterenol sulphate from the Norwegian Medicinal Depot (Harstad, Norway), (±)-epinephrine, (±)-norepinephrine hydrochloride and ascorbic acid from Sigma Chemical Co. (St. Louis, MO). (−)-Propranolol hydrochloride was kindly provided by Imperial Chemical Industries (Cheshire, U.K.). All other chemicals were of analytical grade of purity.

Incubation buffer. NaCl 122 mM, KCl 4.9 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.3 mM and Na_2HPO_4 15.9 mM, pH 7.38.

Serum. Samples of whole blood were obtained from three healthy individuals. After 1 hr at 22°, the samples were centrifuged at 2000 *g* for 20 min and the serum was collected. The three different sera were pooled and then dialyzed for 48 hr at 4° against the incubation buffer with three shifts of buffer, each with a serum–buffer volume ratio of 1 : 20. The serum was frozen (−20°) until used.

AAG. AAG was purified from fresh human serum by the addition of ammonium sulphate (360 g/l) and carrying out the pH adjustments described by Weimer *et al.* [11]. The final product was dialyzed against 25 mM sodium acetate buffer (pH 4.1) and applied to a column of DEAE-Sephadex A-50, previously equilibrated against this buffer. The column was washed with several bed volumes of the above acetate buffer and then with one bed volume of 0.15 M NaCl/25 mM sodium acetate buffer (pH 4.1). The glycoprotein was finally eluted from the column using 0.25 M NaCl/25 mM sodium acetate buffer (pH 4.1) and detected by following the absorbance of the eluate at 280 nm. The protein eluted at this step was dialyzed against water, freeze dried and stored frozen (−20°) until required. The final product appeared

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‡ Abbreviations used: IPR, isoproterenol; NE, norepinephrine; EPI, epinephrine; AAG, *alpha*-1 acid glycoprotein; HSA, human serum albumin; SLP, serum lipoproteins; SDS, sodium dodecylsulphate.

homogeneous in SDS–polyacrylamide gel electrophoresis under reducing conditions.

HSA. Human serum albumin (Cohn fraction V) was obtained from Sigma Chemical Co. (St. Louis, MO). The material was dissolved in 25 mM sodium acetate buffer, pH 4.1 and applied to a column of DEAE-Sephadex A-50 and eluted with the same buffer. Protein passing straight through the column was collected and precipitated by adding ammonium sulphate (350 g/l) to the eluate. The precipitated material, separated by centrifugation, was dissolved in water, dialyzed against water and then freeze dried. SDS–polyacrylamide gel electrophoresis of the final product run under reducing conditions showed no detectable AAG.

Serum lipoproteins. Potassium bromide (293.9 g/l) was added to a serum pool (100 ml) from healthy individuals to obtain a density of 1.195 g/ml [12]. After 45 hr centrifugation at 105,000 *g*, the floating lipoproteins were withdrawn and dialyzed for 48 hr at 4° against the incubation buffer with three shifts of buffer, each with a lipoprotein–buffer volume ratio of 1:50. The lipoprotein fractions were stored at 4° and used within two weeks. The fluorescence properties of the purified fractions [13] were unaffected by storage, thus suggesting little or no denaturation.

Electrophoresis. Analytical SDS–polyacrylamide-gel electrophoresis was performed in gel slabs with dimensions of 0.25 cm × 8.0 cm × 8.0 cm at a constant current of 50 mA per slab with 12.5% polyacrylamide in the separating gel and pH 8.3 in the electrophoresis buffer. M_r values were determined by using the "low molecular weight" calibration kit from Pharmacia AB (Uppsala, Sweden).

Equilibrium dialysis. The binding of IPR, NE and EPI to serum or isolated serum proteins was determined in triplicates by equilibrium dialysis at 37° protected against light with an atmosphere of nitrogen. The protein solution (0.575 ml), containing 0.09 mM ascorbic acid and variable concentrations of the adrenergic substances, was separated from the incubation buffer (0.575 ml) containing 0.09 mM ascorbic acid by a semipermeable membrane (Membrane tubing, A. Thomas, Philadelphia, PA) in Perspex cells. Under the established conditions equilibrium was reached after 3–3.5 hr. In the present study the dialysis was routinely terminated after 4 hr. When the purity of the radiolabelled compounds was assessed by the recommended thin layer chromatography system, during dialysis less than 10% degradation was found.

Ligand concentrations. The concentrations of the adrenergic stimulators were calculated from the distribution of labelled ligand, the concentrations of labelled (IPR: 4 nM, NE: 3 nM and EPI: 4 nM) in absence or presence of unlabelled ligand (IPR: 7 nM–0.8 mM, NE: 3 nM–0.4 mM, EPI: 4 nM–0.4 mM) added and the volumes each side of the dialysis membrane. Radioactivity was determined by scintillation counting (Packard Tri-Carb Spectrometer, Model 3320) using duplicate samples (50 μl) from each side of the dialysis chamber, mixed with 3 ml scintillation liquid (Dilusolve, Packard Instruments, Groeningen, The Netherlands).

Calculations. The binding data were analyzed using the IBM-PC version [14] of the radioligand binding programs: "EBDA, LIGAND" (Elsevier Biosoft, Cambridge, U.K.). The program "LIGAND" was originally written by Munson and Rodbard [15]. The model which gave a random distribution of the points around the fitted line was chosen, and the iterations were continued until a non-significant difference in the binding parameters was obtained. Linear regression analysis was performed using Microstat® (Ecosoft Inc., Indianapolis, IN).

Protein concentration. Protein concentration in serum and in the solutions of isolated proteins was determined according to Lowry *et al.* [16] using bovine serum albumin as standard.

RESULTS

Binding in serum

For catecholamine concentrations of 10–15 nM, the binding in serum (protein concentration: 78.9 ± 3.9 g/l, mean ± SD, N = 3) was 28.8 ± 2.2% for IPR, 25.7 ± 1.7% for NE and 22.2 ± 2.2% for EPI at 37°. With increasing IPR concentrations the binding in serum became saturated and the non-linear Scatchard-plot [17] (Fig. 1a) demonstrated the existence of more than one class of binding sites in serum. Saturation and non-linear Scatchard-plots were also found for the binding of NE (Fig. 1b) and EPI (Fig. 1c). IPR showed the strongest and EPI the weakest binding to serum at all concentrations tested.

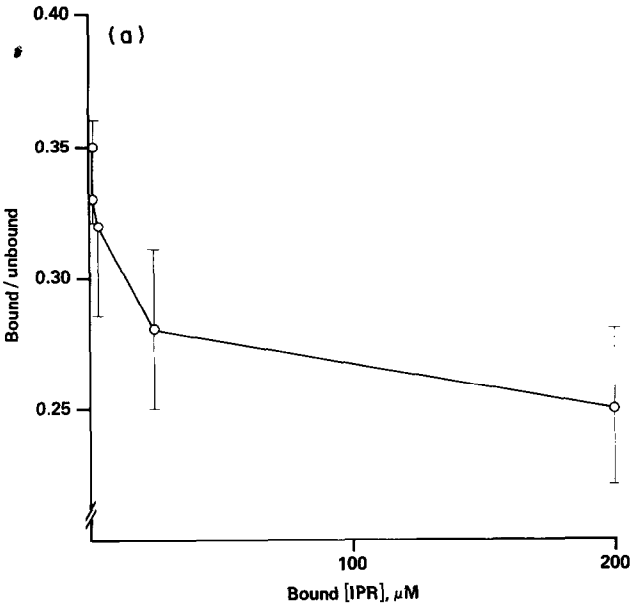
Binding to AAG

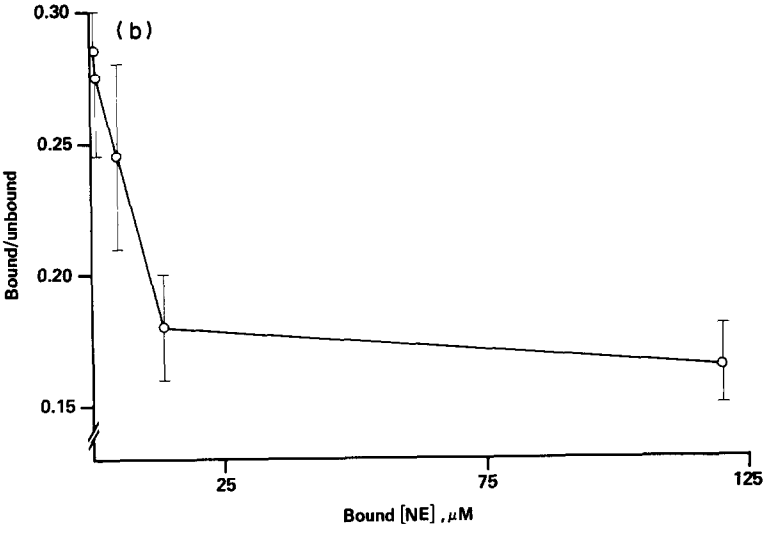
At 37°, the interaction between the catecholamines and AAG showed saturation behaviour, and for all concentrations tested IPR showed the strongest and EPI the weakest binding. When the binding of IPR was plotted according to Scatchard [17], a non-linear binding curve was observed (Fig. 2). Analysis of the binding data showed that each AAG molecule possessed two different classes of saturable binding sites (Table 1). The first class was represented by a single binding site with a relative high affinity and the second class by approximately 10 binding sites with lower affinity. The analysis of the Scatchard-plots for NE and EPI also demonstrated one high affinity and approximately 10 low affinity binding sites per molecule of AAG (Table 1). The various catecholamines exhibited markedly different dissociation constants at the low affinity sites with IPR showing the lowest and EPI the highest dissociation constant. In the case of the single high affinity site, the same order of potency was observed for the binding affinities, but the differences between the dissociation constants were markedly less.

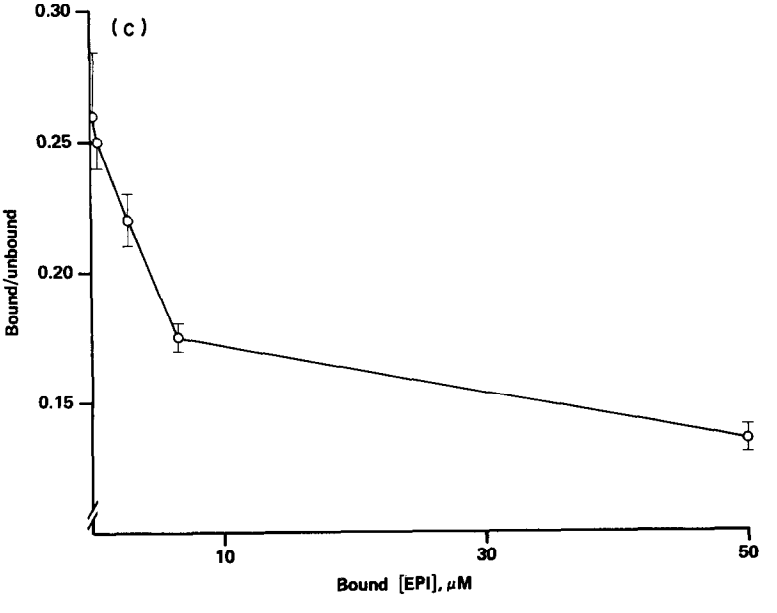
Binding to HSA

For the range of catecholamine concentration tested, the binding of IPR to HSA at 37° did not show saturation behaviour (Fig. 3). When the ratios

Fig. 1. Catecholamine binding in serum (78.4 ± 3.88 g/l) at 37° plotted according to Scatchard (17). (±)-IPR, (b) (±)-NE and (c) (±)-EPI. The results are presented as mean value + and/or − SD for three separate experiments.


(a)
0.40
0.35
0.30
0.25
Bound / unbound
100
200
Bound [IPR], μM


(b)
0.30
0.25
0.20
0.15
Bound/unbound
25
75
125
Bound [NE], μM


(c)
0.30
0.25
0.20
0.15
Bound/unbound
10
30
50
Bound [EPI], μM

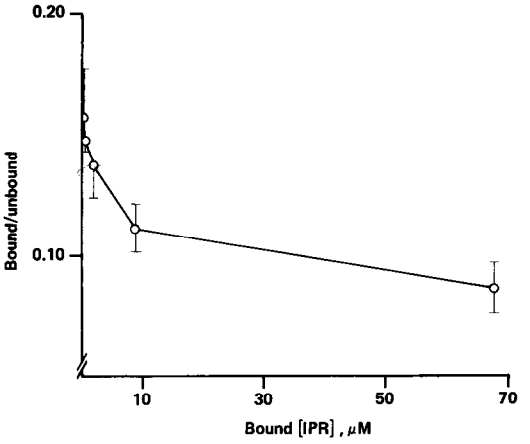


Fig. 2. The binding of (±)-IPR to AAG (17.6 ± 1.9 μM) at 37° plotted according to Scatchard [17]. The results are presented as mean value + and/or − SD from three separate experiments.

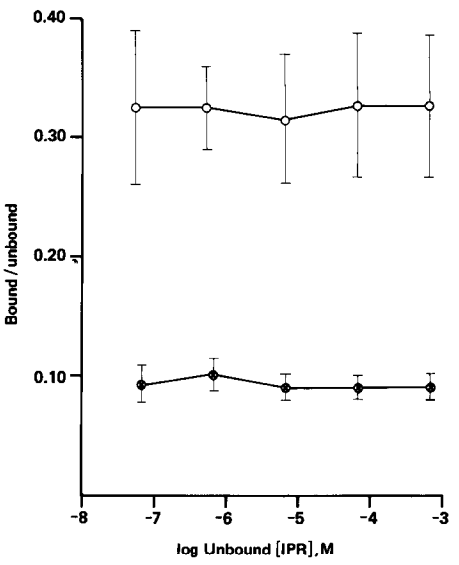


Fig. 3. The binding of (±)-IPR to HSA (555 ± 18 μM) (○—○) and to SLP (7.88 ± 0.16 mg/ml) (⊗—⊗) at 37°. The concentration ratio bound/unbound is plotted as a function of unbound concentration. The results are presented as mean value ± SD from three separate experiments.

between bound and unbound concentrations were correlated to the bound IPR-concentrations in a Scatchard analysis [17], the slopes of the regression lines were not different from zero (Table 2). Furthermore, the binding of NE and EPI to HSA also showed non-saturation type binding under the conditions studied (Table 2). The *y*-axis intercepts of the regression lines indicated that the non-saturable interaction with HSA was strongest for IPR and weakest for EPI (Table 2).

Binding to SLP

At 37°, the binding of IPR to SLP were not saturable for the range of concentrations tested (Fig. 3). In Scatchard analysis [17], the slopes of the regression lines were not different from zero (Table 2). Non-saturability was also found for NE and EPI (Table 2). The differences in the *y*-axis intercepts of the regression lines indicated that IPR interacted strongest and EPI weakest with SLP (Table 2).

The effect of propranolol on IPR binding

The ability of (−)-propranolol (10 nM–0.1 mM) to displace IPR (5–10 μM) bound in serum and to AAG, HSA and SLP was studied. The binding of IPR in serum and to AAG was inhibited in a concentration dependent manner. The (−)-propranolol concentration necessary to displace 50% of bound IPR was 10.8 ± 3.8 μM and 2.4 ± 1.6 μM (mean value ± SD, N = 3) in serum and to AAG, respectively. In contrast, propranolol, even in high concentrations was unable to displace IPR bound to HSA and SLP (results not shown).

DISCUSSION

The stability of catecholamines in plasma [18], has been partly attributed to their binding in plasma [5, 6]. In the present study it was observed that the binding of EPI was 22% for a serum concentration of 10–15 nM. A similar value was obtained for serum concentrations of 0.1–1 μM [5]. The present results (Fig. 1c) show that saturation appears for con-

Table 1. Binding constants for the interaction between (±)-IPR, (±)-NE or (±)-EPI and purified AAG dissolved in the incubation buffer

Ligand	Site 1		Site 2	
	K_d (μM)	B_{max} (μM)	K_d (mM)	B_{max} (μM)
IPR	98.9 ± 11.1	16.0 ± 2.75	2.16 ± 0.076	173 ± 3.22
NE	121 ± 8.72	16.3 ± 6.20	6.47 ± 2.93	211 ± 77.7
EPI	135 ± 16.6	16.2 ± 1.45	14.0 ± 3.78	152 ± 33.0

AAG was purified and the catecholamine binding was determined and analyzed as described in Methods. The results are presented as mean value ± SD from three separate experiments. The protein concentration was 17.6 ± 1.9 μM.

Table 2. The ratios between bound/unbound concentrations (y) were correlated to the bound concentrations (x) of (±)-IPR, (±)-NE and (±)-EPI to HSA and to SLP by linear regression analysis ($y = ax + b$)

Protein/ligand	Slope a (mM^{-1})	y-axis intercept b	r^2	P-values*
HSA/IPR	0.0349 ± 0.0406	0.324 ± 0.0547	0.144 ± 0.146	0.523 ± 0.251
HSA/NE	0.0232 ± 0.0106	0.271 ± 0.0115	0.0846 ± 0.0685	0.663 ± 0.165
HSA/EPI	0.0934 ± 0.239	0.258 ± 0.0138	0.131 ± 0.106	0.626 ± 0.123
SLP/IPR	0.132 ± 0.0874	0.0936 ± 0.00601	0.305 ± 0.110	0.342 ± 0.106
SLP/NE	0.120 ± 0.0597	0.0763 ± 0.00339	0.186 ± 0.141	0.493 ± 0.197
SLP/EPI	0.0865 ± 0.230	0.0459 ± 0.00419	0.253 ± 0.386	0.546 ± 0.406

The bound concentrations ranged from approximately 2 nM to approximately 0.1 mM. The results from each experiment were analyzed and the parameters obtained from three experiments are presented as mean value ± SD. The squared correlation coefficient (r^2) is given since both negative and positive slopes (a-values) were obtained. HSA and SLP were isolated and the binding of the catecholamines was determined as described in Materials and Methods. The protein concentrations were 555 ± 18 μM and 7.88 ± 0.16 mg/ml for HSA and SLP, respectively.

* In all cases $P > 0.1$.

centrations above 1 μM. The identical value for EPI-binding, obtained at 4° [3] is compatible with the observation that EPI-binding in human plasma is independent of temperature in the range 3–37° [6]. The present study demonstrates that the binding of EPI in serum is saturable, in agreement with previous studies in plasma [5, 6]. At lower EPI concentrations gel-filtration methods [6] give markedly higher binding (35–50%) than does equilibrium dialysis (20–24%) as reported previously [5] and in the present work.

The binding of adrenergic stimulators in serum exhibits stereospecificity in the cases of NE [3] and IPR [7]. At physiological concentrations, the binding was 34% for levo-NE and 9% for dextro-NE [3]. These results suggest that the binding of racemic NE would be 20–25%, a prediction in good agreement with the findings in the present study. At physiological NE-concentrations, the extent of binding in plasma from dog was the same (26%) and somewhat lower (19%) in plasma from rabbit [19]. Another study with human plasma demonstrated markedly lower NE-binding (12–13%) for concentrations of 0.1–1.0 μM [5]. One explanation is that partial degradation of labelled NE, forming radioactive products with no or low affinity for plasma proteins, results in an artificial low binding. The long incubation time in absence of both antioxidant and oxygen-free atmosphere could have contributed to such a process. Even if the bound fraction was low, the binding of NE in plasma was saturable [5]. The present results obtained in serum, confirm the saturable behaviour.

It has been reported that 65% of IPR is bound in human plasma [20]. This is in marked contrast to the present value of 29% at similar drug concentrations. Both studies employed equilibrium dialysis at 37° under an oxygen-free atmosphere, but a less physiological buffer, a markedly longer dialysis time and no antioxidant were used in the former [20] compared to the present study. An artificial high binding would occur if quinones were formed due to oxidation of IPR because quinones are highly reactive and may bind irreversibly to plasma proteins [21]. The small differences in dissociation constants for saturable serum binding; IPR: 0.9 mM, NE: 4.6 mM and EPI: 9.8 mM [7] also suggest that the extent of binding of IPR in serum must be similar to that of NE and EPI.

The present observation that propranolol inhibits binding of IPR in serum supports the hypothesis of common adrenergic binding proteins. HSA has been assumed to be the main binding protein in human serum/plasma [1–3], but also other human serum proteins have been reported to bind catecholamines [3, 4]. In rabbit and dog, albumin as well as *alpha-*, *beta-* and *gamma*-globulins have affinity for catecholamines [22, 23].

Alpha-1 globulins in plasma from dog showed the greatest avidity for NE [23], an observation which suggests a binding protein in this plasma fraction. The finding that IPR, NE and EPI inhibit the high affinity binding of propranolol in human serum [7], indicates AAG as a binding protein for catecholamines. In the present study the catecholamine binding to isolated AAG has been characterized. AAG possesses two classes of binding sites for IPR, NE and EPI; one single high affinity site and approximately 10 low affinity sites. IPR had the highest and EPI the lowest affinity for both types of binding sites. In the displacement study [7], the same order of agonist potency was observed, but the affinities were between the present values. One explanation is that the half-maximal inhibitory concentrations of IPR, NE and EPI [7] reflect displacement of propranolol from two different classes of binding sites on AAG. It is well established that propranolol is bound with high affinity (K_d: 1 μM) to a single site on AAG [8–10], but it has been shown that AAG also is able to bind propranolol with low affinity [24, 25]. The results in a previous study [8] could be reinterpreted in terms of one high affinity and five low affinity binding sites on AAG. In the present study the concentration which inhibited maximal IPR binding to AAG was 2.4 μm, probably reflecting displacement both from the high (K_d: 0.7 μM) and low affinity (K_d: 12 μM) binding sites [8]. Taken together

with the present results, this indicates that AAG possess two classes of adrenergic binding sites; a single high affinity site and 5–10 low affinity sites.

A previous study [3] showed that two classes of binding sites are present in the last peak of serum eluted on a Sephadex G-200 column. This serum fraction contains HSA, but also AAG [26]. The affinities of NE and EPI binding to this serum fraction [3] were similar to the present values for binding to isolated AAG. Assuming that HSA was responsible for the high affinity binding, only 5% of the protein molecules possess one binding site. Since the concentration ratio AAG/HSA in serum is approximately 0.05, this indicates that AAG and not HSA is the binding protein in the last eluted peak of serum [3]. The number of low affinity binding sites [3] is more difficult to compare because the non-saturable binding to HSA, shown in the present study, would interfere with the low affinity binding to AAG.

Previously, a catecholamine binding site with an extremely high affinity (K_d: 0.1–0.6 μM) has been reported for plasma [6] and on HSA [2]. The concentration of these binding sites with affinity similar to that reported for beta-adrenergic receptors, was very low. The binding techniques can possibly explain these results [27]. Only 0.03% of the HSA-molecules appeared to have a high affinity catecholamine binding site [2]. However, it has recently been shown that commercial HSA possess various amounts of AAG impurities [28]. In the present work HSA and AAG were highly purified and further analyzed so as to rule out problems arising from contamination of one with the other.

The present study shows that catecholamine interaction with HSA and SLP is non-saturable. Directly, it was demonstrated that the binding ratios of IPR, NE and EPI are constant for all the concentrations tested. This also appears to be a common property among adrenergic agonists and antagonists since it is well established that propranolol is bound in a non-saturable manner to HSA [10, 24, 29, 30] and to SLP [8, 29, 30]. The present study showed that propranolol was unable to inhibit the binding of IPR to HSA and SLP. Taken together, this shows that HSA and SLP lack saturable binding sites, but the possibility of common non-saturable binding domains for adrenergic ligands on HSA and SLP exists. In contrast to the present results, a saturable interaction between catecholamines and VLDL has been reported [3]. The present study shows that the extent of binding to SLP is very low and therefore characterization of the binding to the isolated lipoproteins was not performed.

AAG appears to be an important catecholamine binding protein in serum since specific binding sites exist. Together with HSA and SLP, AAG can account for the total binding in serum, but the possibility of minor contributions from other proteins such as IgM [3] cannot be ruled out. The physiological significance of catecholamine binding in serum is still obscure, but in this connection it should be noted that protein binding has been shown to diminish the biological effect of catecholamines [31].

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