Paul Mugisha · Dirk Gründemann · Edgar Schömig  
Staffan Uhlén

**Binding of [³H]prazosin to α₁A- and α₁B-adrenoceptors, and to a cimetidine-sensitive non-α₁ binding site in rat kidney membranes**

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**Abstract**  
[³H]Prazosin bound to α₁A- and α₁B-adrenoceptors, as well as to a cimetidine-sensitive non-α₁-adrenoceptor binding site in rat kidney membranes. An experimental design is presented where the α₁-adrenoceptors are selectively exposed by blocking the non-α₁ binding site with 60 µM cimetidine. Conversely, the non-α₁ binding site can be selectively exposed by blocking the α₁-adrenoceptors with 600 nM metitepine. The identity of the non-α₁ binding site for [³H]prazosin in the rat kidney, herein pharmacologically characterized by 33 competing substances, is still unknown.

**Keywords**  
[³H]Prazosin · Kidney · Cimetidine · Metitepine · α₁A-Adrenoceptor · α₁B-Adrenoceptor

**Introduction**

The α₁-adrenoceptors are subdivided into the α₁A-, α₁B- and α₁D-subtypes (see Zhong and Minneman 1999). In the rat kidney it has been shown previously that the most frequently used radioligand for α₁-adrenoceptors, [³H]prazosin, binds to the α₁A- and α₁B-subtypes, as well as to a third binding site (Yang et al. 1997). We found that the presence of the non-α₁ binding site made it difficult to accurately quantify the kidney α₁A- and α₁B-adrenoceptors. This obstructs studies involving quantification of kidney α₁A- and α₁B-adrenoceptors after e.g. drug treatments of the rats. In the present study the different binding sites for [³H]prazosin in the rat kidney were characterized pharmacologically. This was done in order to identify specific blockers for the non-α₁ binding site. We found that, if the α₁-adrenoceptors are to be displayed, the non-α₁ binding site could be blocked by cimetidine. Conversely, if the non-α₁ binding site is to be displayed, the α₁-adrenoceptors could be blocked by metitepine.

**Materials and methods**

**Cell culture and HEK293 cell membrane preparations.** The HEK293 cell line stably expressing the authentic rat organic cation transporter rOCT2, and for control the same cell line transfected with vector only (Gründemann et al. 1999), were used. The cells were grown to confluency at 37°C, 5% CO₂, in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal calf serum, in standard tissue culture plastic material as described (Gründemann et al. 1998). Cells were harvested by removing the medium, and adding 6 ml/plate of PBS, including 0.5 mM EDTA, pH 7.4, for 5 min. The cells were then scraped off, and homogenized in 12-ml portions for 12 s by an Ultra-Turrax (IKA T25, equipped with an 8-mm probe). The homogenates were centrifuged at low speed (500 g) for 5 min, in a Beckman J2-21 centrifuge. The resulting supernatants were centrifuged at 38,000 g for 8 min. The pellets were resuspended in 5 ml/plate of 50 mM Tris-HCl, pH 7.5, and these membrane preparations were either used immediately or frozen for use later. When frozen membranes were thawed, they were again ultraturraxed, in order to dissolve precipitated material.

**Kidney membrane preparations.** Kidney membranes were prepared from male Sprague-Dawley rats by homogenizing the excised kidneys by a glass-Teflon homogenizer, and centrifuging, as described above for the HEK293 cells, except that the high-speed centrifugation was performed twice. The final pellets were resuspended in 1.5 mM EDTA, 50 mM Tris-HCl, at pH 7.5 with protein concentrations of about 1.8 mg/ml. Protein concentrations for the kidney membrane preparations were measured according to Lowry et al. (1951), with the inclusion of SDS (Markwell et al. 1978).

**Radioligand binding studies.** Radioligand binding was performed as described (Uhlén and Wikberg 1991) by incubating membranes, containing about 180 µg of protein, in 150 µl of 33 mM Tris-Cl, at pH 7.5 with [³H]prazosin and drugs for 1 h at room temperature and then filtering and washing on Whatman GF/C filters. All assays were performed in duplicate. Multi-curve modelling of radioligand binding data was performed as described earlier (Uhlén and Wikberg 1991), using the BindAid radioligand binding analysis package (Wan System, Umeå, Sweden). Most tests included several competition curves that had been obtained with or without masks with a site-selective drug. The data of all curves included in such a test were subjected to simultaneous analysis in the com-
puter. The formula used to calculate the occupancy at binding sites for $[^3H]prazosin in the presence of masking drugs was:

$$B_t = B_{max} \times \frac{[C_t]}{[K_d] + [C_t]/[K_m] \times \frac{[C_t]}{[C_t] + [C]}},$$

where $C_t$ indicates radioligand and $C$ competitor. In the saturation experiments on $\alpha_1$-adrenoceptors, the non-specific binding was defined by the binding of $[^3H]prazosin in the presence of 20 $\mu$M WB4101 (60 $\mu$M cimetidine was present in all these assays to block the non-$\alpha_1$ site. The average $K_d$ value of $[^3H]prazosin for the $\alpha_1$- and $\alpha_2$-adrenoceptors obtained and used in the calculations of the $K_i$ values of competing drugs for the $\alpha_1$- and $\alpha_2$-adrenoceptors was 0.35 nM (see Results). In the saturation experiments on the non-$\alpha_1$ site, the non-specific binding was defined by the binding of $[^3H]prazosin in the presence of 60 $\mu$M cimetidine (600 $nM metitepine was present in all these assays to block the $\alpha_1$-adrenoceptors). The $K_d/K_i$ value of $[^3H]prazosin/prazosin obtained and used in the calculations of the $K_i$ values of competing drugs for the non-$\alpha_1$ binding site present in the kidney was 167 $nM (see Results). Because of the low affinity of $[^3H]prazosin for the non-$\alpha_1$ binding site, the experimental IC$_{50}$ values of the competing drugs for the non-$\alpha_1$ binding site become close to their corresponding calculated $K_i$ values (and theoretical $K_d$ values; the Cheng-Prusoff equation states that $K_d=IC_{50}/(1+[C]/[K_m]);$ in the present study $[C]$ was about 2 $nM and $K_d$ about 167 $nM$). For the unknown native binding site in HEK293 cells, also showing low affinity for $[^3H]prazosin, only IC$_{50}$ values were calculated. It can be noticed that in the experiments on kidney membranes where $[^3H]prazosin binds to $\alpha_1$-adrenoceptors, the non-$\alpha_1$ binding site, and non-specific binding, the fraction bound/free of the radioligand at 2 $nM$ was about 7% [representing 3% of the free radioligand binding to the non-$\alpha_1$ binding site (see Fig.3A), 3% to the $\alpha_1$-adrenoceptors, and 1% to the non-specific binding]. This fraction of bound should be low enough to avoid interpretation problems due to depletion of the radioligand. The binding constants of competing drugs are given as the negative logarithm of the dissociating constants ($pK_i$ values or $pIC_{50}$ values). Experimentally determined values are given as the arithmetic means $\pm$ SEM. Linear correlation analysis of $pK_i$ values was performed in DeltaGraph Pro 3.5, from DeltaPoint. The figures were constructed using DeltaGraph Pro 3.5.

Materials. $[7$-methoxy-$[^3H]prazosin (74 $Ci/mmole$) was from New England Nuclear (NEN) through DuMedical (Stockholm, Sweden). AR2329 (2-(2,4-($O$-methoxyphenyl))-piperazin-1-yl)-ethyl-4,4-dimethyl-1,3(2$H$, 4$H$)-isquinolindione), HEAT (also known as BE2254; 2-[[4-(4-hydroxyphenyl)ethy1]aminomethyl]-1-terttralone) and Tocris-451 (3-[2-[4-(chlorophenyl)piperazin-1-yl]-ethyl]pyrimidol-5,4-b-hindole-2,4-dione) were from Tocris Cookson (Bristol, UK), Adenosine, BMY7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decan-7,9-dione), guanabenz, metitepine, 5-methyl-ureapidil, WB4101 (2-(2,6-dimethoxynonyethyl)-aminomethyl-1,4-benzodioxane) were from Research Biochemicals (Natick, Mass., USA). Amiloride, corticosterone, cyclosporin A, deccynium22 (1,1’-diethyl-2,2’-cyanine iodide), desipramine, fluphenazine, pargyline, prazosin, progesterone, quinidine, tropolone and vinblastine were from Sigma (St. Louis, Mo., USA). Cimetidine was from Smith Cline and French (Welwyn Garden City, UK). Clonidine was from Boehringer-Ingelheim (Ingelheim/Rhein, Germany). Doxazosin was from Pfizer (Täby, Sweden). Thioridazine was a gift from Sandoz (Basel, Switzerland). Labetalol and ranitidine were from Glaxo (Greenford, UK). Phentolamine was from Ciba-Geigy (Basel, Switzerland). Verapamil was from Knoll (Ludwigshafen, Germany). Reserpine was from Pharmacia (Uppsala, Sweden). All other chemicals were from Sigma and were of analytical quality.

**Results**

As can be seen in Fig. 1A–C, the competition curves of metitepine, cimetidine and prazosin at the sites labeled by $[^3H]prazosin in the presence of $[^3H]prazosin in the rat kidney membranes. Membranes were incubated with 2 $nM[^3H]prazosin, and competition curves for test drugs (as indicated) were obtained, either for the test drug alone (■), in the presence of 60 $\mu$M cimetidine (to block the non-$\alpha_1$ binding site) (▲), or in the presence of 600 $nM metitepine (to block the $\alpha_1$-adrenoceptors) (●). The _curved lines_ represent the computer-drawn multi-curve fit obtained from the simultaneous fitting of all data in each experiment to a model that assumed that ligands bound to two sites according to the law of mass action. One-hundred percent on the y-axis represents the amount of $[^3H]prazosin bound in the absence of competing drugs. Shown are the results of single representative experiments.

2 $nM[^3H]prazosin in the rat kidney were clearly biphasic. This indicates that $[^3H]prazosin labeled $\alpha_1$-adrenoceptors, as well as an unknown binding site in the kidney membranes.

The high affinity site for metitepine represents the $\alpha_1$-adrenoceptors (see Wikberg-Matsson et al. 1998). Accordingly, in an experimental design aiming at displaying the non-$\alpha_1$ binding site, the $\alpha_1$-adrenoceptors could be