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Autoradiographic localization of β-adrenoceptor subtypes in guinea-pig kidney

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1 The distribution of β-adrenoceptor subtypes in slide-mounted sections of guinea-pig kidney has been examined by the technique of *in vitro* labelling combined with autoradiography.

2 Binding of (−)-[^{125}I]-cyanopindolol (Cyp) to kidney sections equilibrated and dissociated slowly, was saturable and stereoselective with respect to the isomers of propranolol and pindolol. These characteristics were appropriate for binding to β-adrenoceptors.

3 Delineation of β-adrenoceptor subtypes was achieved by use of betaxolol (β_1-adrenoceptors) and ICI 118,551 (β_2-adrenoceptors) and computer assisted curve fitting techniques. Both β_1- and β_2-adrenoceptors were present in the proportions 1:2.

4 ^{3}H-Ultrofilm images of (−)-[^{125}I]-Cyp binding to guinea-pig kidney sections showed localized patches of binding in the cortex and concentrated binding in the outer stripe of the medulla. Cortical receptors were of the β_1 subtype and those associated with the outer stripe of the medulla were of the β_2-adrenoceptor subtype. β_1-Adrenoceptors were concentrated over glomeruli and β_2-adrenoceptors over the straight portion of the proximal tubule.

Introduction

The mammalian kidney is well innervated by the sympathetic nervous system. The renal nerves follow the renal arteries and form terminal varicosities which are particularly dense around blood vessels, the juxtaglomerular apparatus, proximal and distal tubules (Barajas & Wang, 1979). It is well established that noradrenaline released from the varicosities by stimulation of the nerves acts at β-adrenoceptors to produce an increase in renin secretion (Johnson *et al.*, 1971; Coote *et al.*, 1972; Ganong, 1973; Johns & Singer, 1974; Johnson *et al.*, 1976; Taher *et al.*, 1976). β-Adrenoceptors may also play a role in increasing fluid reabsorption and decreasing sodium excretion (Bello-Reuss, 1980; Hollingsead & Willis, 1980) although the main control by the sympathetic nervous system also appears to be through α-adrenoceptors which in the dog mediate increases in sodium and water reabsorption in the absence of changes in glomerular filtration rate, renin release or renal blood flow (Osborn *et al.*, 1983). It has been suggested that there are few β-adrenoceptors associated with the renal vasculature (Bomzon *et al.*, 1975; Buckley *et al.*, 1979), since infusion of β-adrenoceptor agonists or antagonists has no effect on renal blood flow. Biochemical studies of the distribution of β-adrenoceptor-stimulated adenylate cyclase activity in isolated nephron segments show considerable variations in distribution between species. No β-adrenoceptor-stimulated adenylate cyclase activity has been reported in proximal tubules, the thin limb of the loop of Henle, or the medullary portion of the thick ascending limb of the loop of Henle (Morel *et al.*, 1981). However, the cortical portion of the thick ascending limb shows marked activity in the rat, less in mouse and is absent in the rabbit. β-Adrenoceptor-stimulated adenylate cyclase activity is present in rat distal tubule, collecting tubule and cortical collecting duct whereas in the rabbit it is present only in the latter two areas. Only in the rabbit is there any activity found in the medullary collecting duct.

Renal β-adrenoceptors have also been identified by use of the radioligand binding technique. Studies with [^{3}H]-(−)-dihydroalprenolol or [^{125}I]-hydroxybenzylpindolol indicated stereospecific binding sites with the molecular characteristics of β-adrenoceptors (Gavendo *et al.*, 1980; Woodcock & Johnston, 1980). Although these receptors appear to be of the β_1-

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subtype, more recent studies using membranes prepared from rat renal cortex and another radioligand [^{125}I]-cyanopindolol (Cyp) show that both β_1- and β_2-adrenoceptors are present (Snavely *et al.*, 1982). Membranes prepared from isolated glomeruli have markedly enhanced numbers of β-adrenoceptors which are mainly of the β_1-subtype (McPherson & Summers, 1983).

A number of different techniques have been used in attempts to localize β-adrenoceptors in the kidney. The fluorescent derivative of propranolol, 9 amino-acridin propranolol has been used to try and identify β-adrenoceptors (Atlas *et al.*, 1977). However, this ligand binds to β-adrenoceptors with relatively low affinity and it is likely that the sites identified are associated with drug excretion (Hess, 1979; Cornett & Meizel, 1980). Immunohistochemical techniques have indicated alprenolol binding sites associated with afferent and efferent arterioles, juxtaglomerular apparatus, proximal and distal tubules but not with glomeruli, loop of Henle and collecting tubules (Amenta *et al.*, 1983). Direct autoradiographic localization of β-adrenoceptors with radioligands indicates a rather different picture with high concentrations of receptors associated with glomeruli, distal and cortical collecting tubules (Summers & Kuhar, 1983; Munzel *et al.*, 1984; Lew & Summers, 1984; Lew *et al.*, 1984a).

In the study described here, autoradiographic techniques have been used to localize β-adrenoceptor subtypes in guinea-pig kidney. The validity of the sites identified has been verified by examining the kinetics, stereospecificity and pharmacological properties of the binding in slide-mounted tissue sections prepared in an identical manner to the autoradiographic specimens (Kuhar, 1981). A preliminary report of some of this work has appeared (Lew *et al.*, 1984a).

Methods

Radioiodination of (−)-cyanopindolol

(−)-Cyp was radio-iodinated with Na^{125}I by a procedure modified from that described by Engel *et al.* (1981). The following constituents were added sequentially to a polypropylene test tube at room temperature (22–25°C); 15 μl of 10 mM HCl containing 10 mM (−)-cyanopindolol, 30 μl 0.3 M KH_2PO_4 pH 7.6, 3 mCi Na^{125}I and 30 μl aqueous solution of chloramine T (0.34 mg ml^{-1}) thoroughly mixed and allowed to stand for 15 min. Sodium metabisulphite (1 mg ml^{-1}, 300 μl) was added to stop the reaction. The mixture was made alkaline with 15 μl 1 M NaOH, extracted with 300 μl ethylacetate (containing 0.01% phenol) and the top phase (containing the (−)-[^{125}I]-Cyp) collected using a Pasteur pipette. This extraction procedure was repeated 3 times before combining the extracts. (−)-[^{125}I]-Cyp was purified by descending paper chromatography using Whatman 3 MM (4 × 40 cm) paper with 0.1 M ammonium formate (containing 0.01% phenol) pH 8.5 as the solvent carrier. After 2–3 h the chromatogram was removed, cut into 1 cm strips and the iodinated ligand was extracted with methanol. (−)-[^{125}I]-Cyp forms a single peak (R_F = 0.09) while (−)-Cyp occurs further down the chromatogram (R_F = 0.53). (−)-[^{125}I]-Cyp was stored in methanol at − 20°C for up to 2 months.

General procedure

Kidneys from guinea-pigs (500–800 g) of either sex were perfused *in situ* with buffer consisting of 0.32 M sucrose and Krebs phosphate (composition in mM: NaCl 119, KCl 4.8, $MgSO_4$ 1.2, NaH_2PO_4 10.0, $CaCl_2$ 1.27) pH 7.6 (1:1), and then the same solution containing 0.1% formaldehyde. The kidneys were quickly excised, and frozen in isopentane previously cooled in liquid N_2. Sections (10 μm) were cut on a Tissue Tek II Cryostat and mounted onto subbed microscope slides (Young & Kuhar, 1979). The incubation medium consisted of 170 mM Tris HCl pH 7.6, 10 μM phenylmethylsulphonyl fluoride (PMSF) and 0.01% ascorbate. Sections were incubated for 150 min at room temperature (22–25°C). The concentrations of (−)-[^{125}I]-Cyp used were 50 pM in biochemical studies and 20–50 pM for autoradiography. Following incubation, labelled sections were quickly rinsed in 170 mM Tris HCl pH 7.6, followed by 2 × 15 min washes in the same medium and finally rinsed in distilled water (22–25°C). For biochemical assessment of renal β-adrenoceptors, sections were wiped and counted in a LKB multigamma counter. In autoradiographic studies, sections were dried by cold dehumidified air and stored at 4°C in sealed boxes containing silica gel. Non-specific binding was defined by co-incubation with 1 μM (−)-propranolol or 200 μM (−)-isoprenaline.

^{3}H-Ultrofilm autoradiography

To determine the distribution of (−)-[^{125}I]-Cyp binding sites in guinea-pig kidney, labelled sections were placed in close contact with LKB ^{3}H-Ultrofilm, in light-tight boxes or X-ray cassettes (Kodak X-omatic) and exposed for 3–5 days. The ^{3}H-Ultrofilm was developed using Kodak D 19, briefly rinsed in water, and fixed with Kodak Rapid fix.

Histochemical procedure

After photographic development, sections were washed in Krebs-phosphate buffer for 20 min and histologically fixed for 10–15 min in acetone:McIl-

wain's buffer (3:2) pH 5.2. Sections were then stained in naphthol-AS-MX-phosphate reagent containing fast blue RR salt (0.5 mg ml^{-1}). After the development of a satisfactory blue colouration in the renal cortex the sections were counterstained with pyronine Y (1 min). After mounting in PVP medium, sections were viewed under an Olympus BHT 2 microscope.

Analysis of results

Estimation of the dissociation constant (K_D) of (−)-[^{125}I]-Cyp, the maximum number of binding sites (B_{max}) and parameters from competition studies were performed using the computer programme 'EBDA' (McPherson, 1983) in combination with the iterative curve fitting programme 'LIGAND' (Munson & Rodbard, 1980).

Drugs and chemicals

Drugs and chemicals were obtained from the following sources: Na^{125}I (Amersham International); (−)-cyanopindolol, (−)- and (+)-pindolol (Sandoz, Basle); (−)- and (+)-propranolol, ICI 118,551 erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-01, (ICI Australia Ltd.); betaxolol (Synthelabo); naphthol-AS-MX-phosphate, fast blue RR (Sigma). All other chemicals were of analytical grade.

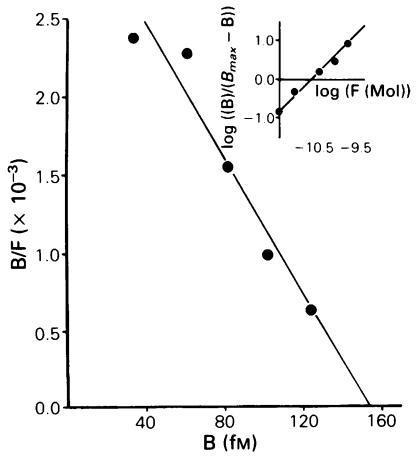

Figure 1 Representative Hill and Scatchard plots of (−)-[^{125}I]-cyanopindolol (Cyp) binding to microscope slide-mounted guinea-pig sections. Scatchard plot shows that (−)-[^{125}I]-Cyp binds to a single population of sites with high affinity. (B_{max} = 4.58 fmol/section, K_D = 46.1 pM.) Inset: Hill plot demonstrating linearity and lack of cooperativity of (−)-[^{125}I]-Cyp binding (nH = 0.98).

Results

Characterization of [^{125}I]-cyanopindolol binding to slide-mounted sections of guinea-pig kidney

Before it can be concluded that autoradiographic localization of binding is to β-adrenoceptors it is necessary to establish that these sites have the required molecular characteristics. Studies were therefore carried out to determine the biochemical characteristics of the binding of (−)-[^{125}I]-Cyp to microscope slide-mounted sections of guinea-pig kidney. At room temperature (22–25°C) (−)-[^{125}I]-Cyp associated slowly with the receptor, as equilibrium occurred only after 2.5 h. Dissociation after addition of 1 μM (−)-propranolol was extremely slow, only 30% of the ligand dissociating after 16 h. After incubation, sections were rinsed then washed twice for 15 min in 170 mM Tris HCl pH 7.6, followed by a final rinse in distilled water before counting or autoradiographic processing. The washing procedure markedly improved the signal to noise ratio without affecting specific binding.

Saturation of (−)-[^{125}I]-cyanopindolol binding

Binding of (−)-[^{125}I]-Cyp to slide-mounted sections of guinea-pig kidney was saturable and to a single population of non-interacting sites as shown by Scatchard and Hill plots in Figure 1. Scatchard plots were linear and gave values of B_{max} (maximum no. of binding sites) of 4.96 ± 0.49 fmol/section (n = 4) and K_D (dissociation constant, − 1/gradient Scatchard plot) of 45.2 ± 4.0 pM (n = 4). Hill plots were linear with a gradient of 0.99 ± 0.01 (n = 4) indicating a lack of cooperativity in binding.

Stereoselectivity of (−)-[^{125}I]-cyanopindolol binding

Slide-mounted sections of guinea-pig kidney were incubated with (−)-[^{125}I]-Cyp (50 pM) with and without a series of concentrations of the stereoisomers of propranolol and pindolol. For both β-adrenoceptor antagonists binding was highly stereoselective as shown in Figure 2. All isomers displaced (−)-[^{125}I]-Cyp binding with slope factors close to unity (Table 1). The (−)-isomers of propranolol and pindolol were respectively, 122 and 51 times more effective in competing for (−)-[^{125}I]-Cyp binding than the (+)-isomers.

Resolution of β-adrenoceptor subtypes in guinea-pig kidney

The subtype selective β-adrenoceptor antagonists betaxolol (β_1-adrenoceptors) and ICI 118,551 (β_2-adrenoceptors) were used to delineate the β_1- and β_2-adren-

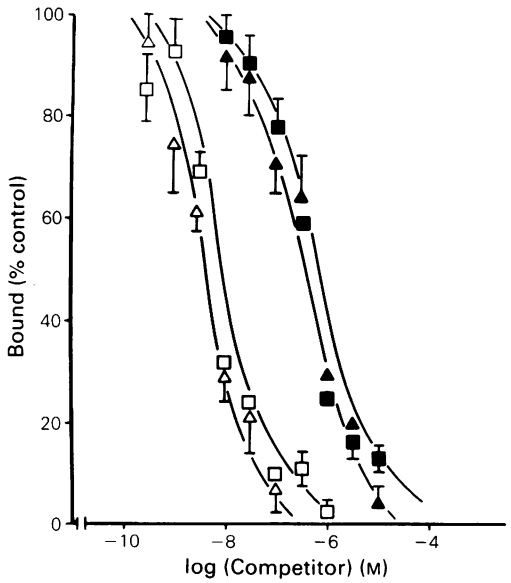

Figure 2 Competition for (−)-[^{125}I]-cyanopindolol (Cyp) binding sites by (−)-(△) and (+)-propranolol (▲), (−)-(□) and (+)-pindolol (■) in microscope slide-mounted guinea-pig kidney sections. Sections were incubated with (−)-[^{125}I]-Cyp (50 pM) and the indicated concentrations of the isomers of propranolol and pindolol. Points are the mean of 4 experiments; vertical lines show s.e.mean. The (−)-isomers of propranolol and pindolol were, respectively, 122 and 51 times more effective in competing for (−)-[^{125}I]-Cyp binding sites than the (+)-isomers (see Table 1).

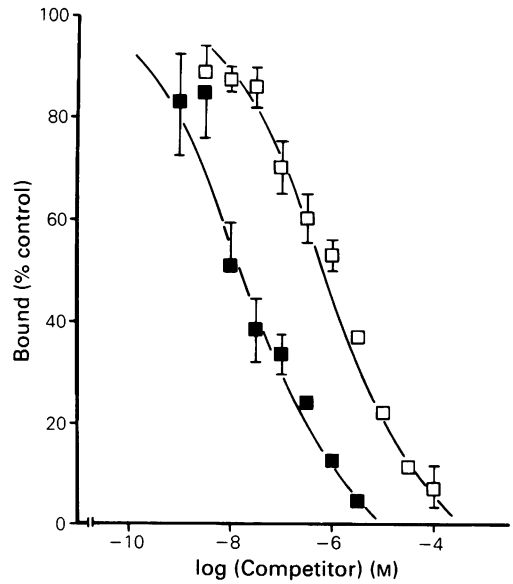

Figure 3 Competition for (−)-[^{125}I]-cyanopindolol (Cyp) binding sites on guinea-pig kidney sections by the subtype selective β-adrenoceptor antagonists, betaxolol (β_1, □) and ICI 118,551 (β_2, ■). Sections were incubated with (−)-[^{125}I]-Cyp (50 pM) and the antagonists at concentration shown on the abscissa scale. Points are the mean of 8 (betaxolol) and 4 (ICI 118,551) experiments conducted in triplicate; vertical lines show s.e.mean.

oceptor populations. In biochemical experiments both compounds produced competition curves for (−)-[^{125}I]-Cyp binding which had low slope factors (0.52–0.55) (Figure 3). This is behaviour typical of selective antagonists competing for binding to 2 or more sites. These curves could be resolved using the iterative curve fitting programme 'LIGAND' (Munson & Rodbard, 1980). The β_1-selective antagonist betaxolol bound to a high affinity site with a K_D of 28 nM (β_1-adrenoceptors) and a low affinity site with a K_D of 1300 nM (β_2-adrenoceptors). The β_2-selective antagonist ICI 118,551 identified a high affinity site with a K_D of 6.9 nM (β_2-adrenoceptors) and a low affinity site with a K_D of 152 nM (β_1-adrenoceptors) (Table 2). β_2-Adrenoceptors were about twice as numerous as β_1-adrenoceptors. The values of K_D for the high affinity site for each subtype selective

Table 1 Stereoselectivity of (−)-[^{125}I]-cyanopindolol binding to slide-mounted guinea-pig kidney sections

Drug	K_D(nM)	*Slope* (pseudo nH)	n
(−)-Propranolol	2.7 ± 0.8	− 0.81 ± 0.10	4
(+)-Propranolol	332 ± 77	− 1.0 ± 0.14	4
(−)-Pindolol	3.5 ± 0.4	− 0.96 ± 0.13	4
(+)-Pindolol	179 ± 51	− 0.82 ± 0.12	4

Values are means ± s.e.mean.
Values of K_D and slope factors were evaluated using 'EBDA' (McPherson, 1983) and 'LIGAND' (Munson & Rodbard, 1980).

Table 2 Competition for (−)-[^{125}I]-cyanopindolol binding by subtype selective β-adrenoceptor antagonists

Drug	K_{D1} (nM)	K_{D2} (nM)	*% total* B_{max_1}	B_{max_2}	*Slope* (pseudo nH)*	n
Betaxolol	28	1300	38.3	61.7	− .55 ± .07	8
ICI 118,551	6.9	152	75.9	24.1	− .52 ± .08	4

* Values are means ± s.e.mean
Values of K_{D1}, K_{D2}, B_{max1}, and B_{max2}, were evaluated using 'LIGAND' (Munson & Rodbard, 1980) and nH by 'EDBA' (McPherson, 1983).

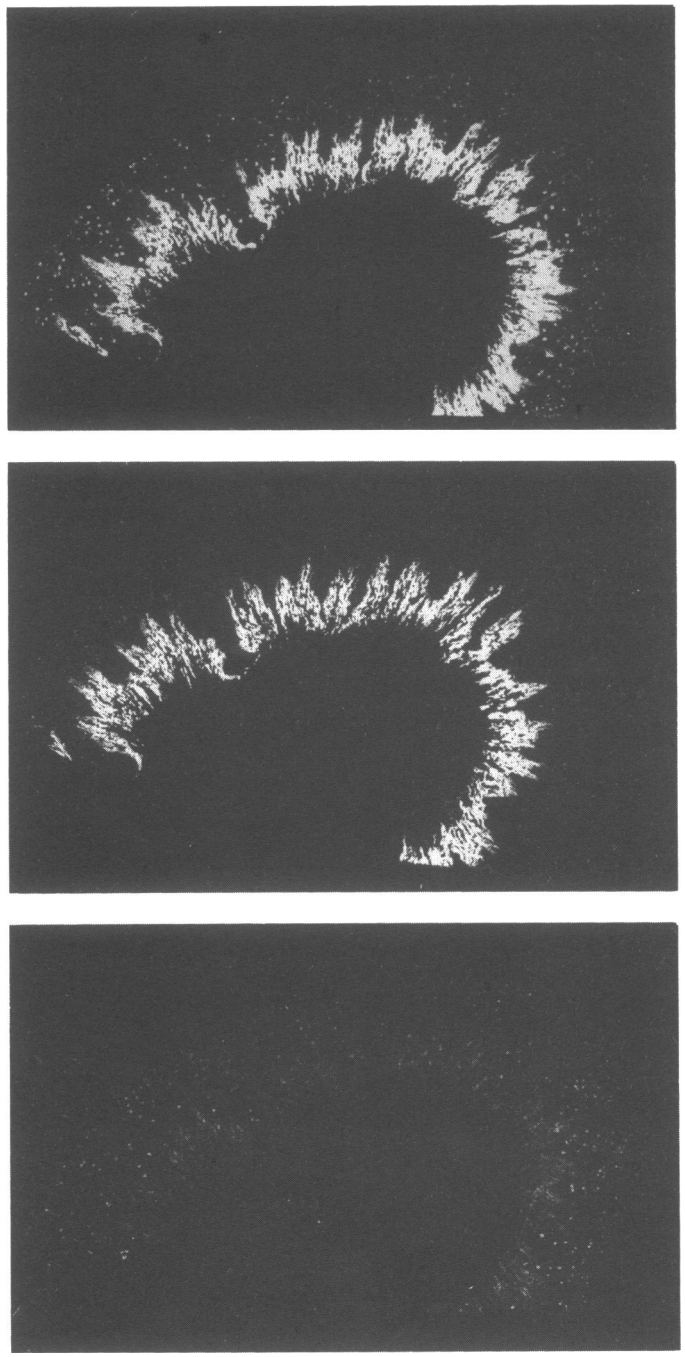

Figure 4 Photographs taken directly from ^{3}H-Ultrofilm images after apposition to microscope slide-mounted guinea-pig kidney sections. Micrographs are from sections incubated either with (a) (−)-[^{125}I]-cyanopindolol (Cyp) (20 pM) alone or together with (b) betaxolol, (500 nM), or (c) ICI 118,551, (50 nM). In (a) localized areas of binding are observed in the cortex and associated with the outer stripe of the medulla but not in the inner medulla. In (b) cortical binding could be selectively abolished by the β_1-antagonist betaxolol whilst in (c) that in the outer stripe of the medulla was abolished by the selective β_2-antagonist ICI 118,551.

antagonist were used as a guide in choosing suitable concentrations to block selectively β_1- and β_2-adrenoceptors in the autoradiographic studies.

Autoradiographic studies of the distribution of β-adrenoceptors using ^{3}H-Ultrofilm

Sections labelled with (−)-[^{125}I]-Cyp placed in apposition to ^{3}H-Ultrofilm produced clear images which demonstrated the distribution of β-adrenoceptors in kidney sections. The ^{3}H-Ultrofilm images were used as negatives to produce Figure 4. The total β-adrenoceptor population is concentrated in two areas. High concentrations were found in the outer stripe of the medulla and small localized patches in the cortex (Figure 4a). The image of the cortical receptors was selectively abolished by the inclusion of 500 nM betaxolol ($18 \times K_D$) in the labelling medium (Figure 4b). In contrast, the image produced by binding to receptors in the outer stripe of the medulla was selectively abolished by 50 nM ICI 118,551 ($7 \times K_D$) (Figure 4c). Only very faint images were obtained over sections incubated with (−)-[^{125}I]-Cyp and (−)-propranolol (1 μM) or (−)-isoprenaline (200 μM). These studies indicate that the cortical receptors are largely of the β_1- and those in the outer stripe of the medulla of the β_2-adrenoceptor subtype.

Discussion

In previous studies it has been shown that binding sites for (−)-[^{125}I]-Cyp with the molecular characteristics of β-adrenoceptors are found in rat kidney. These receptors are largely of the β_1-subtype and are localized to glomeruli and distal and cortical collecting tubules (Summers & Kuhar, 1983; Summers *et al.*, 1984). Receptors of the β_2-subtype are less numerous and have a more diffuse distribution.

In the present study it has been established that the biochemical characteristics of (−)-[^{125}I]-Cyp binding to slide-mounted sections of guinea-pig kidney are those of β-adrenoceptors. Thus the binding was stereoselective with respect to the isomers of propranolol and pindolol, saturable, reversible (albeit slowly) and to a single population of non-interacting sites. The slow reversibility of (−)-[^{125}I]-Cyp binding agrees with a previous report (Hoyer *et al.*, 1982). Problems were not experienced with (−)-[^{125}I]-Cyp binding to non β-adrenoceptor sites as occurs in mouse kidney studied under identical conditions (Petrovic *et al.*, 1983; Lew *et al.*, 1984b). In guinea-pig kidney, nonspecific binding determined either with (−)-propranolol (1 μM) or (−)-isoprenaline (200 μM) gave precisely the same levels determined either biochemically or autoradiographically. However, in mouse kidney binding was completely abolished by (−)-propranolol but not by (−)-isoprenaline and autoradiograms prepared using the latter displacer showed intense binding in the medulla indicating the presence of a lipid related binding site for (−)-[^{125}I]-Cyp in this species (Lew & Summers, unpublished). The subtype selective antagonists betaxolol (Boudot *et al.*, 1979; Dickinson *et al.*, 1981) and ICI 118,551 (Bilski *et al.*, 1980; Dickinson *et al.*, 1981) produced shallow competition curves which could be resolved clearly into two components. These studies indicated that β_1- and β_2-adrenoceptors are present in guinea-pig kidney sections in a ratio of 1:2. As in rat kidney the β-adrenoceptors associated with glomeruli were of the β_1-subtype. In contrast however, the β_2-adrenoceptor population in the guinea-pig kidney was highly localized to the straight portion of the proximal tubule identified by a positive reaction for alkaline phosphatase (Wachstein & Bradshaw, 1965). In addition there was no evidence in the guinea-pig of binding to distal or cortical collecting tubules. These studies indicate that there is variation in the distribution of renal β-adrenoceptors between species. It is of interest that variations in the distribution of β-adrenoceptor stimulated adenylate cyclase are also observed in the isolated nephron segments of different species (Morel *et al.*, 1981).

The functional correlates of these receptors is only now becoming clear. In glomeruli there are a number of processes controlled by β-adrenoceptors. Renin release from the juxtaglomerular apparatus (JGA) is stimulated by renal nerve stimulation and administration of β-adrenoceptor agonists. In the dog (Kopp *et al.*, 1980; Osborne *et al.*,) and rat (Campbell *et al.*, 1979; De Saulles *et al.*, 1979; Nakane *et al.*, 1980) these receptors are of the β_1-subtype. However, a consistent observation in autoradiographic studies of β-adrenoceptors in the kidney (Summers & Kuhar, 1983; Lew & Summers, 1984; Lew *et al.*, 1984a) is that the receptors are present over the entire glomerular portion of the section. This may indicate that there are β-adrenoceptors controlling other processes associated with the glomerulus or that renin production is not confined to the JGA. β-Adrenoceptors located on the epithelial cells of the glomerular tuft (Busuttil *et al.*, 1971) or on the mesangial cells are believed to have a role in the control of erythropoetin production (Fink & Fisher, 1976). However, the limited pharmacological evidence available indicates that these receptors are of the β_2-subtype in rabbits in contrast to the β_1-adrenoceptors observed in the present experiments. It would be of interest to examine the effect of the highly selective β_1- and β_2-adrenoceptor antagonists on erythropoetin production in the light of our observations.

The other main area of β-adrenoceptor localization was in the straight portion of proximal tubules. It is not clear whether these β_2-adrenoceptors are linked to

adenylate cyclase but in rat kidney there was a remarkably good correlation between the distribution of β-adrenoceptors determined autoradiographically (Summers & Kuhar, 1983) and β-adrenoceptor stimulated adenylate cyclase activity (Morel *et al.*, 1981). These receptors could be involved in fluid and electrolyte transport (Besarab *et al.*, 1977).

There was no evidence in the present studies for β-adrenoceptors (either β_1 or β_2) associated with the guinea-pig renal vasculature. This would agree with previous functional studies in which the infusion of β-adrenoceptor agonists or antagonists had no effect on renal blood flow (Bomzon *et al.*, 1975; Buckley *et al.*, 1979).

In conclusion, this study demonstrates the presence in guinea-pig kidney of high concentrations of β_1-adrenoceptors localized in glomeruli and β_2-adrenoceptors localized to the straight portion of the proximal tubule.

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