Pharmacological characterization and anatomical localization of prejunctional β -adrenoceptors in the rat kidney

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1 The subtype and anatomical localization of β -adrenoceptors mediating facilitation of stimulus-induced overflow of noradrenaline ('prejunctional β -adrenoceptors') are not conclusively known to date. The present study was undertaken to characterize these receptors by use of pharmacological methods as well as to define their localization (prejunctional or postjunctional) with radio-ligand binding and autoradiography techniques combined with surgical denervation of the sympathetic innervation to the rat kidney.

2 Exposure of the kidney to (–)-isoprenaline, the nonselective β -adrenoceptor agonist, resulted in a dose-dependent facilitation of stimulus-induced neurotransmitter overflow. This response was inhibited by propranolol, the β_1 - and β_2 -adrenoceptor antagonist, with a pA_2 of 9.20 suggesting that the prejunctional β -adrenoceptors are not of the β_3 -subtype.

3 The rank order of potency and potency ratios of β -adrenoceptor agonists at renal prejunctional β -adrenoceptors (EC_{50} for agonist/ EC_{50} for (–)-isoprenaline) were: (–)-isoprenaline (1) > procaterol (2) > salbutamol (3) > adrenaline (10) > (+)-isoprenaline (25). However, dobutamine, the β_1 -adrenoceptor agonist, failed to enhance stimulus-induced overflow of noradrenaline. These results are indicative of the presence of β_2 -adrenoceptors as prejunctional β -adrenoceptors.

4 Facilitation elicited by (–)-isoprenaline and procaterol, the selective β_2 -adrenoceptor agonist, was inhibited by ICI 118,551, the selective β_2 -adrenoceptor antagonist, with pK_b values of 9.20 and 9.35, respectively at renal prejunctional β -adrenoceptors. Similarly, the pK_b values of metoprolol, the selective β_1 -adrenoceptor antagonist, at renal prejunctional β -adrenoceptors were determined to be 6.25 and 6.18 against (–)-isoprenaline and procaterol, respectively. These results suggest the presence of a homogeneous population of β_2 -adrenoceptors as prejunctional β -adrenoceptors.

5 Radio-ligand binding analysis of renal β -adrenoceptors revealed the prevalence of the β_1 -subtype as compared to the β_2 -subtype (63% vs 37%). However, surgical denervation of the rat kidney, resulting in more than 90% reduction in renal noradrenaline content, selectively reduced the β_2 -adrenoceptor population by 80%, implying the presence of β_2 -adrenoceptors on renal sympathetic nerve terminals.

6 Autoradiographic analysis demonstrated the presence of β_1 -adrenoceptors on cortical structures such as glomeruli and tubules. β_2 -Adrenoceptors were found to be present on tubules (minor population), collecting tubules in outer medulla and the adventitia and adventitial-medial border of intraparenchymal branches of the renal artery. Surgical denervation of the rat kidney resulted in the disappearance of β_2 -adrenoceptors associated with the intraparenchymal branches, without affecting the β -adrenoceptor populations at other sites. These results support the notion that the β_2 -subtype is present on renal sympathetic nerve terminals and demonstrate that these prejunctional β_2 -adrenoceptors are associated with the renal vasculature and not with renal tubules.

7 The results of the present investigation demonstrate that renal prejunctional β -adrenoceptors are of the β_2 -subtype in nature. These receptors are present on sympathetic nerve terminals which are associated with the renal vasculature.

Keywords: Autoradiography; β -adrenoceptor; characterization; facilitation; prejunctional; rat kidney; sympathetic neurotransmission

Introduction

Regulation of sympathetic neurotransmission is achieved at various levels in the body. An important regulatory site is the sympathetic nerve terminal which is presumably endowed with various 'prejunctional' receptors. Activation of these receptors results in either inhibition or facilitation of exocytotic noradrenaline (NA) release from the nerve terminals. One of the facilitatory receptor systems is the prejunctional β -adrenoceptor system.

Prejunctional β -adrenoceptors enhance stimulus-induced (S-I) overflow of NA from sympathetic varicosities. The presence of these receptors has been demonstrated in various tissues and species (cf. Misu & Kubo, 1986). It has been

suggested that physiologically, prejunctional β -adrenoceptors may be activated by neuronally released NA (Adler-Graschinsky & Langer, 1975). Alternatively, adrenaline may assume the role of physiological activator for these receptors (Stjarne & Brundin, 1976). However, the question of physiological agonist for prejunctional β -adrenoceptors is intimately related to the β -adrenoceptor subtype mediating the facilitatory response because, NA is equipotent with or more potent than adrenaline at the β_1 - and β_3 -subtypes while adrenaline is more potent than NA at the β_2 -subtype (Lands *et al.*, 1967; Bond & Clarke, 1988).

Attempts have been made to characterize prejunctional β -adrenoceptors, but have yielded contradictory results. For example, the involvement of β_1 -adrenoceptors in the facilitatory response was implied in the cat hindlimb (Dahlof *et*

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al., 1975), rabbit ear artery (Majewski & Rand, 1981) and rat portal vein (Ortiz de Urbina *et al.*, 1992). Conversely, β_2 -adrenoceptors were suggested to mediate the facilitatory response in the human omental artery and vein (Stjarne & Brundin, 1976), rat mesentery (Kawasaki *et al.*, 1982) and guinea-pig papillary muscle (Valenta & Singer, 1991). Biological variation between different tissues and species cannot account for these controversial results, as different groups have reported different prejunctional β -adrenoceptor subtypes in the same tissue (Dahlof *et al.*, 1978; Westfall *et al.*, 1979; Remie *et al.*, 1988; Ortiz de Urbina *et al.*, 1992). Instead, the primary reason for these conflicting results seems to be the use of inadequate methods (such as, using only one or two concentrations of the β -adrenoceptor agents or failing to achieve equilibrium conditions) instead of appropriate pharmacological techniques, to characterize the receptors. Thus, a proper characterization of prejunctional β -adrenoceptors with the aid of suitable pharmacological methods is warranted.

Another controversial issue surrounding peripheral prejunctional β -adrenoceptors is their anatomical localization. Although these receptors are believed to be present on sympathetic nerve terminals (hence 'prejunctional'), anatomical evidence supporting this concept is sparse to date. The prejunctional localization of these receptors has been reported in a few tissues, such as the rat and cat spleen (Sharma & Banerjee, 1978; Dahlof *et al.*, 1986), while supportive evidence has been difficult to obtain in other tissues, such as the rat kidney cortex (Fortin & Sundaresan, 1989). In addition, it has been proposed recently that β -adrenoceptors mediating facilitation of S-I overflow of NA in the rat vena cava may be postjunctionally located and may elicit facilitation by releasing a transjunctional mediator (Gothert & Kollacker, 1986), thus raising a question regarding the concept of prejunctional localization of 'prejunctional' β -adrenoceptors. Also, even in tissues where the prejunctional localization of these receptors has been reported (Sharma & Banerjee, 1978; Dahlof *et al.*, 1986), the precise anatomical location of the sympathetic varicosities containing these receptors within the tissue has not been examined.

Therefore, the present investigation was undertaken to characterize prejunctional β -adrenoceptors in the rat kidney by use of pharmacological techniques such as pK_b determination for antagonists and determination of potency ratio of agonists. The putative prejunctional localization of these receptors also was studied by examining β -adrenoceptor populations in the innervated and surgically denervated preparations by radio-ligand binding and autoradiography.

Methods

Isolated perfused rat kidney preparation

Surgical procedure The rat kidney was isolated as described previously (Schwartz & Eikenburg, 1988). Male Wistar rats (Charles-River, PA, U.S.A.) weighing 250–300 g were anaesthetized with sodium pentobarbitone (60 mg kg⁻¹, i.p.). The abdominal cavity was opened by a midline incision and the right kidney, right renal artery, superior mesenteric artery and aorta were located and cleaned of surrounding tissues. Fifteen minutes following heparinization (1500 i.u., i.v.), the superior mesenteric artery was cannulated and then the cannula was advanced further into the renal artery. The right kidney along with the renal artery was isolated from the rat, placed in the perfusion apparatus and perfused (6 ml min⁻¹) with carbogenated Krebs bicarbonate solution (pH: 7.4) maintained at 37°C. The composition of the Krebs bicarbonate solution in mM was: KH₂PO₄ 1.2, NaCl 118.4, KCl 4.8, NaHCO₃ 25.0, glucose 5.0, MgSO₄ 7H₂O 1.2, Na₂S₂O₅ 0.1, EDTA 0.03 and CaCl₂·2H₂O 2.5. Cocaine (10 μ M) and corticosterone (40 μ M) were added to the solution in order to inhibit neuronal and extraneuronal uptake, respectively. A bipolar platinum electrode was placed around the renal

artery for periarterial nerve stimulation. Perfusion pressure was monitored by a pressure transducer connected to the perfusion system and recorded on a Grass polygraph. Any preparation in which perfusion pressure changed by more than 5 mmHg in the absence of any stimulation was discarded, though this happened very rarely.

Experimental protocol Following a 30 min stabilization period, a priming stimulus was delivered (480 pulses, 4 Hz, 1 ms, supramaximal V) after which the α -adrenoceptor antagonist, phentolamine (10 μ M) was added to the perfusate to minimize the influences of prejunctional α -adrenoceptors and S-I perfusion pressure changes on S-I NA overflow. Captopril (0.1 μ M) and indomethacin (3 μ M) also were added to the perfusate to inhibit renal synthesis of angiotensin II and prostaglandins, respectively. The kidney was allowed to stabilize for an additional 45 min after which 9 consecutive stimulations (S1 to S9) were delivered at 20 min intervals using a Grass stimulator (10 pulses, 1 Hz, 1 ms, supramaximal V). During each stimulation and 1 min following that, the perfusate was collected and analyzed for NA content. At the end of the experiment, the kidney was weighed and homogenized with 5 ml of 1.0 N HClO₄ and an aliquot of the homogenate supernatant was analyzed for determination of the total renal NA content. The weight of kidney ranged from 1.5 to 2 g.

Estimation of NA NA was extracted from the perfusate and homogenate samples by an alumina extraction method (Schwartz & Eikenburg, 1988) and the content in the final eluate was analyzed by reverse phase high pressure liquid chromatography coupled with electrochemical detection (h.p.l.c.-e.c.). The overflow of NA in the absence of stimulations was non-detectable.

Calculation and expression of NA release S-I release of NA has been expressed as absolute and fractional overflows. These terms were calculated using the following formulae:

$$\text{Absolute overflow (pmol g}^{-1}\text{)} = \frac{\text{Stimulus-induced overflow (pmol)}}{\text{Kidney weight (g)}}$$

$$\text{Fractional overflow (\%)} = \frac{\text{Absolute overflow (pmol g}^{-1}\text{)}}{\text{Renal NA content at the time of stimulation (pmol g}^{-1}\text{)}} \times 100$$

When a stimulation was performed in the presence of a β -adrenoceptor agonist, facilitation of S-I overflow of NA was observed which was calculated as:

$$\text{Facilitation (\%)} = \frac{(\text{Fractional overflow}_{\text{Agonist}}) - (\text{Fractional overflow}_{\text{Control}})}{\text{Fractional overflow}_{\text{Control}}} \times 100$$

Here, Fractional overflow_{Agonist} = Fractional overflow in the presence of the agonist

Fractional overflow_{Control} = Fractional overflow in the absence of the agonist

Fractional overflow of NA in the absence of agonist was calculated by taking an average of the fractional overflow at S1 and S2.

Construction of agonist concentration-response curve On the basis of preliminary experiments, an appropriate agonist concentration-range (over which the responses are concentration-dependent) was chosen and 6 to 7 concentrations of the agonist, half a log unit apart, were selected from this range to generate a concentration-response curve. It was ensured that the maximum effective concentration of the agonist was used in each experiment. The first 2 stimulations (S1 and S2) were carried out in the absence of an agonist. The agonist was added to the perfusion fluid at a fixed time (2 min or 10 min) prior to each stimulation beginning with S3 and remained until the end of the stimulation. Agonist was

added in the order of increasing concentrations from S3 to S9 and the facilitatory response at each concentration was measured to generate a concentration-facilitatory response curve. A single concentration-response curve was generated in each experiment.

When agonist concentration-facilitatory response curves were generated in the presence of various concentrations of an antagonist, an appropriate concentration of the antagonist was added to the perfusion fluid 45 min prior to S1 and was perfused throughout the experiment. The agonist concentration-response curve was generated in the presence of the antagonist as described above.

Fitting of agonist concentration-response curve The concentration-response curve to an agonist was fitted to a logistic equation using a computer programme (Kaleidagraph, Abelbeck Software). The concentrations producing 50% of the maximum response (EC_{50} values) were obtained directly from the equation. The logistic equation which was used to best fit the curves is as follows (Waud & Parker, 1971):

$$\text{Response} = \frac{M \times [A]^n}{[A]^n + (EC_{50})^n}$$

Here, M = maximum response; $[A]$ = molar agonist concentration; n = slope of the curve; EC_{50} = agonist concentration producing half the maximum response.

Determination of rank order of potency and potency ratio The rank order of potency and the potency ratios for β -adrenoceptor agonists relative to (–)-isoprenaline were determined. For the potency ratio, the EC_{50} value for the agonist was divided by the EC_{50} value for (–)-isoprenaline. Thus, any potency ratio greater than 1 indicates that the agonist is that many fold less potent than (–)-isoprenaline.

Determination of pA_2 and pK_b values In some cases when only one concentration of the antagonist was used, the pA_2 value was calculated from the Furchgott equation (Furchgott, 1972) which is as follows:

$$pA_2 = \log ([A']/[A] - 1) - \log [B]$$

Here, $[A'] = EC_{50}$ in the presence of the antagonist; $[A] = EC_{50}$ in the absence of the antagonist; $[B]$ = molar antagonist concentration.

In other cases, Schild regression analysis was performed to determine the pK_b values of the antagonists. This analysis is based upon following equation (Arunlakshana & Schild, 1959):

$$\log (\text{concentration ratio} - 1) = \log [B] - \log K_b$$

Here, $[B]$ = molar antagonist concentration, K_b = equilibrium dissociation constant of the antagonist.

Once the criteria of similar maximum responses and parallelism of the agonist concentration-response curves in the absence and presence of the antagonist was satisfied (Kenakin, 1984), Schild regression analysis was carried out. The concentration-ratio (shift) of the agonist concentration-response curve in the presence of a particular concentration of the antagonist as compared to that in the absence of the antagonist was calculated by dividing the EC_{50} value obtained in the presence of the antagonist by the EC_{50} value obtained in the absence of the antagonist. Then a Schild plot was obtained by plotting log molar concentrations of the antagonist on the abscissa scale and log (concentration ratio – 1) on the ordinate scale. The data points were fitted by simple regression and the slope of the regression line was determined. If the slope was not significantly different from 1 then a line with the slope of 1 was best fitted to the points (MacKay, 1978) and was extrapolated to determine the x-intercept at $y = 0$. This x-intercept was accepted as the pK_b value for the antagonist at the receptors.

Radio-ligand binding and autoradiography studies

Animal and tissue treatment Twenty male Wistar rats of 235–265 g were anaesthetized with pentobarbitone (60 mg kg^{-1} , i.p.) and the right renal artery was exposed. In 10 rats, the right kidney was denervated by stripping the right renal artery surgically and swabbing it with a 10% phenol solution dissolved in ethanol. Ten other rats were sham-operated. Animals were allowed to survive for one week after denervation and then were killed by pentobarbitone overdose. The right kidney was dissected out and washed with an ice-cold 0.9% NaCl solution to remove blood and cell debris. The upper half of the right kidney was weighed and used for NA assay. The lower half of the kidney was cut into 0.3 cm slices which were frozen in isopentane cooled with liquid nitrogen and used for β -adrenoceptor assay and autoradiography. Sections, 8 μm thick, were obtained with a -20°C microtome cryostat and mounted on pre-weighed gelatin-coated microscope slides.

NA assay The upper half of the right kidney was homogenized in ice-cold 0.1 M HClO_4 (5 ml g^{-1} tissue). The homogenate was centrifuged at 2000 g for 20 min in a refrigerated centrifuge and the resulting supernatant was used for NA assay. NA was assayed by h.p.l.c.-e.c. according to the procedure detailed elsewhere (Amenta *et al.*, 1987).

Radio-ligand binding Sections from surgically denervated or sham-operated kidneys were preincubated for 30 min at room temperature in Krebs bicarbonate solution. Then they were incubated in the same solution for 120 min at room temperature with increasing concentrations of radiolabelled iodo-cyanopindolol ($[^{125}\text{I}]\text{-CYP}$) (0.05 to 1 nM). Selective binding to the different β -adrenoceptor subtypes was evaluated by incubating parallel sections in the presence of different concentrations of ICI 89,406 (selective β_1 -adrenoceptor antagonist) or ICI 118,551 (selective β_2 -adrenoceptor antagonist) (Bilski *et al.*, 1983; Engel *et al.*, 1985). The optimal binding time and temperature were determined in a series of preliminary experiments. Nonspecific binding was evaluated by incubating some sections in the presence of $1 \mu\text{M}$ (–)-propranolol. Furthermore, to assess the specificity of $[^{125}\text{I}]\text{-CYP}$ binding to β -adrenoceptors, other sections were incubated in the presence of different concentrations of (–)- and (+)-propranolol, isoprenaline, NA and phentolamine.

After incubation, the sections were washed in cold buffer (2 times, 5 min each), rapidly rinsed in distilled water, wiped onto Whatman glass filters and placed in scintillation vials. Vials were then placed in a gamma counter (LKB, Bromma, Sweden) for radioactivity determination. Further details of the radio-ligand binding procedures are reported elsewhere (Amenta *et al.*, 1991).

Autoradiography For autoradiographic experiments, the sections were incubated according to the optimal binding parameters determined in preliminary experiments using a radio-ligand concentration of 0.4 nM. Such a concentration produced the highest specific/nonspecific binding ratio, as determined in radio-ligand binding experiments (see results). The selective antagonists ICI 89,406 and ICI 118,551 were used at concentrations of 100 nM and 70 nM, respectively, on the basis of radio-ligand binding data. At the end of the incubation, the sections were washed as above, rinsed quickly in distilled water and air dried. Then they were post-fixed by exposure to formaldehyde vapours at 80°C for 30 min and processed for autoradiography according to the method of Young & Kuhar (1979). Briefly, coverslips coated with Ilford L4 nuclear emulsion (diluted 1:1 with distilled water) were attached to the slides; then they were exposed for 3–6 days in light-proof boxes, developed with Kodak D19, fixed with Agefix-Agfa, stained with toluidine blue and observed under a light microscope equipped with both bright- and dark-field condensers.

The density of silver grains developed by autoradiography and representing [125 I]-CYP binding sites was assessed according to the procedure detailed in an earlier paper (Amenta *et al.*, 1991). Briefly, silver grains were counted in an area of $500 \mu\text{m}^2$ as determined by an image analyzer connected via a TV camera to the microscope, using a final magnification at the camera of $\times 400$. Counts were made by three investigators independently on at least 5 consecutive sections per kidney ($n = 10$, denervated or sham-operated).

Statistical evaluation

Fractional overflows of NA in different groups in the absence and presence of various antagonist concentrations but in the absence of agonist (at S1 and S2) were compared by unpaired *t*-test or one factor analysis of variance. Maximum facilitation in different groups was compared by an unpaired *t*-test or one factor analysis of variance. To test parallelism of concentration-response curves, each average concentration-response curve was converted to a straight line by transforming the values of mean facilitatory response at each concentration into probits which were plotted on the ordinate scale against log molar concentration of the agonist on the abscissa scale. The slopes of the straight lines were then compared by analysis of covariance. The slope of the Schild regression plot was tested by *t*-test to determine whether it was significantly different from unity. In the radio-ligand binding and autoradiography studies, the densities of [125 I]-CYP binding among the various groups were compared by one factor analysis of variance (ANOVA). A significance level of $P < 0.05$ was accepted.

Drugs and chemicals

Chemicals used in preparation of the Krebs bicarbonate solution and elsewhere were reagent or h.p.l.c. grade and obtained from standard sources. The following stock solutions were divided into aliquots and stored at -80°C . Solutions of (–)-isoprenaline (Sigma), (+)-isoprenaline (> 99%, Sigma) and (–)-adrenaline (Sigma) were prepared in 0.01 N hydrochloric acid containing 1 mM sodium metabisulphite.

Figure 1 Concentration-facilitatory response curves to (–)-isoprenaline in the absence (○) and presence of 30 nM propranolol (●), $n = 5$. Data are presented as mean $\pm$ s.e.mean. The maximum facilitatory responses were $93 \pm 6\%$ and $107 \pm 14\%$ in the control and propranolol groups, respectively. The fractional overflows of noradrenaline (NA) in the absence of (–)-isoprenaline were $0.58 \pm 0.03\%$ and $0.57 \pm 0.06\%$ while the renal NA contents were 495 ± 83 and $435 \pm 23 \text{ pmol g}^{-1}$ in control and propranolol groups, respectively. These experiments were performed in the presence of cocaine ($10 \mu\text{M}$) and corticosterone ($40 \mu\text{M}$) to inhibit neuronal and extraneuronal uptake, phentolamine ($10 \mu\text{M}$) to block prejunctional α -adrenoceptors, indomethacin ($3 \mu\text{M}$) to inhibit renal prostaglandin synthesis and captopril ($0.1 \mu\text{M}$) to inhibit renal angiotensin II synthesis.

Solutions of dobutamine (Eli Lilly), procaterol (Sigma), metoprolol (Sigma) and ICI 118,551 (Imperial Chemical Industries) were prepared in distilled water. The following stock solutions were stored at 4°C . A solution of indomethacin (Sigma) was prepared in ethanol. Solutions of cocaine (Sigma) and corticosterone (Sigma) were prepared in 0.01 N hydrochloric acid and 90% dimethylsulfoxide, respectively. All stock solutions were diluted with Krebs bicarbonate solution to the appropriate concentrations on the day of the experiment. A fresh stock solution of salbutamol (Sigma) was prepared daily and was diluted with Krebs bicarbonate solution to the appropriate concentrations. Captopril (Sigma) and phentolamine mesylate (Ciba-Geigy) were weighed daily, dissolved in distilled water and added to Krebs bicarbonate solution. [125 I]-cyanopindolol (specific activity $2,000 \text{ Ci mmol}^{-1}$) was purchased from the Radiochemical Centre of Amersham (Buckinghamshire, UK).

Results

Control experiments performed in the absence of an agonist demonstrated that S-I fractional overflow of NA was reproducible during the course of the experiment (data not shown). The β -adrenoceptor antagonists, when given alone, did not affect S-I fractional overflow of NA (data shown in legends to figures representing agonist concentration-response curves in the absence and presence of the antagonist).

Effects of the non-selective β -adrenoceptor agonist, isoprenaline, on neurotransmitter overflow

Exposure of the kidney to the non-selective β -adrenoceptor agonist, (–)-isoprenaline, results in dose-dependent facilitation of S-I neurotransmitter overflow (Figure 1). The tissue was exposed to each agonist concentration for 2 min to avoid desensitization of prejunctional β -adrenoceptors (Lakhlani & Eikenburg, 1993). The maximum facilitatory response was $93 \pm 6\%$ while the EC_{50} concentration was 1.2 nM (Figure 1). The concentration-effect curve to (–)-isoprenaline was shifted 52 fold to the right in a parallel fashion by propranolol, the β_1 - and β_2 -adrenoceptor antagonist, without significantly altering the maximum facilitatory response ($107 \pm 14\%$) (Figure 1). The pA_2 for propranolol at prejunctional β -adrenoceptors, determined from the Furchgott equation (Furchgott, 1972), was estimated to be 9.2.

The rank order of potency and the potency ratios of β -adrenoceptor agonists

As a first step in identifying the β -adrenoceptor subtype responsible for the facilitatory effect on neurotransmitter overflow, the rank order of potency and the potency ratios of various β -adrenoceptor agonists were determined. An agonist exposure time of 2 min was used. The observed rank order of potency was (–)-isoprenaline > procaterol > salbutamol > adrenaline > (+)-isoprenaline (Figure 2a). In addition, procaterol, salbutamol, adrenaline and (+)-isoprenaline were observed to be 2-fold, 3-fold, 10-fold and 25-fold less potent than (–)-isoprenaline, respectively (Table 1). It should be noted here that adrenaline, a mixed β - and α -adrenoceptor agonist, did not affect the perfusion pressure (data not shown), suggesting that α -adrenoceptor blockade by phentolamine remained effective in the presence of adrenaline.

In contrast to the agonists described above, dobutamine, the β_1 -adrenoceptor agonist, did not produce any effect on sympathetic neurotransmission in the rat kidney (Figure 2b) within the concentration range of 1 nM to $10 \mu\text{M}$. Dobutamine, also an α -adrenoceptor agonist at higher concentrations, did not influence perfusion pressure within this concentration-range, suggesting that α -adrenoceptor blockade by phentolamine remained effective. At the concentrations of $100 \mu\text{M}$ and 1 mM , dobutamine produced marked vasoconstriction,

Figure 2 (a) The concentration-facilitatory response curves to (–)-isoprenaline (O), procaterol (●), salbutamol (□), adrenaline (■) and (+)-isoprenaline (Δ) at prejunctional β -adrenoceptors, $n = 3-5$. Data are presented as mean $\pm$ s.e.mean. The maximum facilitatory responses for (–)-isoprenaline, procaterol, salbutamol, epinephrine and (+)-isoprenaline were $93 \pm 6\%$, $96 \pm 6\%$, $99 \pm 3\%$, $115 \pm 11\%$ and $104 \pm 27\%$, respectively. The fractional overflows of noradrenaline (NA) in the absence of agonist were $0.58 \pm 0.03\%$, $0.40 \pm 0.10\%$, $0.36 \pm 0.05\%$, $0.48 \pm 0.05\%$ and $0.53 \pm 0.06\%$ while renal NA contents were 495 ± 80 , 601 ± 54 , 511 ± 4 , 484 ± 46 and 389 ± 51 pmol g^{-1} in (–)-isoprenaline, procaterol, salbutamol, adrenaline and (+)-isoprenaline groups, respectively. For other experimental conditions, refer to Figure 1. (b) Stimulus-induced fractional overflow of NA in the absence (C) and presence of the β_1 -adrenoceptor agonist, dobutamine, $n = 4$. The data are presented as mean $\pm$ s.e.mean. The renal NA content was 442 ± 21 pmol g^{-1} . For other experimental conditions, refer to Figure 1.

suggesting its action at postjunctional (and probably at prejunctional) α -adrenoceptors (data not shown). Hence, its effect on renal sympathetic neurotransmission at these concentrations was not evaluated.

Determination of the pK_b for antagonists at prejunctional β -adrenoceptors

In the first series of experiments, ICI 118,551 was used as a representative of selective β_2 -adrenoceptor antagonists and its pK_b was determined with (–)-isoprenaline and procaterol, the selective β_2 -adrenoceptor agonist, to activate the facilitatory β -adrenoceptors. A β_1 -adrenoceptor agonist could not be used for reasons explained above. When an agonist exposure time of 2 min or 5 min was used in these experiments, an apparent noncompetitive behaviour by ICI 118,551 was observed (data not shown). When agonist exposure time was increased to 10 min in these experiments, parallel shifts indicative of competitive antagonism were observed. Therefore, an agonist exposure time of 10 min was used in all experiments. The appropriate antagonist concentration was added to the perfusate 45 min prior to the first stimulation and was present for the remainder of the experiment.

When concentration-facilitatory response curves to (–)-isoprenaline were generated in the absence and presence of ICI 118,551 (3–30 nM), the maximum facilitatory responses observed in the different groups were not significantly different (Figure 3a and Table 2). The curves were parallel and suggested a competitive antagonism by ICI 118,551 (Figure 3a). The slope of the regression line from the Schild plot was not significantly different from unity and when constrained to a slope of 1 the pK_b value was determined to be 9.20 (Figure 3b and Table 2). Similarly, ICI 118,551 (3–30 nM) produced parallel dextral shifts of the procaterol concentration-facilitatory response curves. The maximum response to procaterol was not significantly affected by any concentration of ICI 118,551 (Figure 3c and Table 2). When the Schild analysis was performed, the slope of the regression line was not significantly different from unity and when constrained to a slope of one the pK_b value for ICI 118,551 against procaterol was determined to be 9.35 (Figure 3d).

In a second series of experiments, the pK_b of the selective β_1 -adrenoceptor antagonist, metoprolol, was determined with (–)-isoprenaline and procaterol. Metoprolol produced parallel dextral shifts of the isoprenaline concentration-facilitatory response curves without significantly affecting the maximum responses (Figure 4a and Table 2). Schild analysis revealed that slope of the regression line was not significantly different from unity and when the plot was constrained to a slope of unity, the pK_b value of metoprolol against (–)-isoprenaline was 6.25 (Figure 4b and Table 2). Similarly, when the effects of ICI 118,551 on the procaterol concentration-facilitatory

Table 1 Potency ratios of β -adrenoceptor agonists relative to (–)-isoprenaline at prejunctional β -adrenoceptors, β_1 -adrenoceptors and β_2 -adrenoceptors

Agonist	Potency ratio ^a		
	Prejunctional β -adrenoceptors ^b	β_2 -adrenoceptors	β_1 -adrenoceptors
(–)-Isoprenaline	1	1	1
Procaterol	2	0.5 to 0.14 ^c	127 to 1000 ^e
Salbutamol	3	2 to 6 ^d	170 to 2500 ^d
Adrenaline	10	20 to 33 ^e	4 to 8 ^e
(+)-Isoprenaline	25	46 ^f	35 ^f

^aPotency ratio was calculated by dividing EC_{50} value for the agonist by EC_{50} value for (–)-isoprenaline. Thus, any potency ratio greater than 1 indicates that the agonist is that many fold less potent than (–)-isoprenaline while any potency ratio less than 1 indicates that the agent is more potent than (–)-isoprenaline.

^bPotency ratios of the agonists at prejunctional β -adrenoceptors were derived from the data presented in Figure 4a.

^cYabuuchi (1977); O'Donnell & Wanstall (1985).

^dFarmer *et al.* (1970); O'Donnell (1972).

^eLands *et al.* (1967); Furchgott (1972).

^fBowman & Rand (1982).

response curves were examined, ICI 118,551 produced parallel dextral shifts of the procaterol curves without significantly altering the maximum response (Figure 4c and Table 2). The Schild analysis of the metoprolol-procaterol data resulted in a regression line with a slope that was not significantly different from unity and when a line with the slope of 1 was best fitted to the points, the pK_b value of metoprolol at prejunctional β -adrenoceptors was determined to be 6.18 (Figure 4d).

NA levels in sham-operated and denervated rat kidneys

The results of the NA assay in control (sham-operated) or surgically denervated kidneys are summarized in Table 3. Surgical kidney denervation reduced the renal NA content by more than 90%. This indicated the effectiveness of the denervation procedures.

Radio-ligand binding

[125 I]-CYP binding to sections of the rat kidney was time-, temperature- (data not shown) and concentration-dependent (Figure 5a). The plateau in specific binding was reached at a [125 I]-CYP concentration of about 0.4 nM; K_d value was 0.20 nM and B_{max} was 108 fmol mg^{-1} tissue (Figure 5a). About 81% of [125 I]-CYP binding to sections of rat kidney was specific (Figure 5a). Experiments performed using the selective β_1 - and β_2 -adrenoceptor antagonists, ICI 89,406 and ICI 118,551, respectively, revealed that ICI 89,406 was effective in inhibiting [125 I]-CYP binding at lower concentrations than ICI 118,551 (Figure 5b). The analysis of binding to each β -adrenoceptor subtype demonstrated a prevalence of the β_1 -subtype as compared to the β_2 -subtype (63% vs 37%) (Table 3).

Surgical denervation of the rat kidney resulted in a 44% reduction in the total β -adrenoceptor density and an 80% reduction in the β_2 -adrenoceptor sites (Table 3). In contrast, β_1 -adrenoceptors were not significantly affected.

Autoradiography

Silver grains developed by autoradiography and corresponding to [125 I]-CYP binding sites could be detected both in kidney cortex and outer medulla, as well as intraparenchymal renal blood vessels. The incubation in the presence of the two specific antagonists allowed for discrimination between the two β -adrenoceptor subtypes.

In cortical structures (glomeruli and renal tubules), β_1 -adrenoceptors were dominant (Figure 6). Incubation in the presence of ICI 89,406, however, demonstrated the presence

Figure 3 (a) and (c) Concentration-facilitatory response curves to (-)-isoprenaline (a) and procaterol (c) in the absence (●) and presence of 3 nM (○), 10 nM (■) and 30 nM (□) ICI 118,551, $n = 4-6$. Data are presented as mean $\pm$ s.e.mean. The following values are for control, ICI 118,551 (3 nM), ICI 118,551 (10 nM) and ICI 118,551 (30 nM) groups, respectively: Fractional overflow of noradrenaline (NA) in the absence of (-)-isoprenaline - $0.33 \pm 0.05\%$, $0.32 \pm 0.03\%$, $0.29 \pm 0.06\%$ and $0.34 \pm 0.05\%$; renal NA content in (-)-isoprenaline group - 388 ± 31 , 424 ± 74 , 419 ± 38 and 485 ± 63 pmol g^{-1} ; fractional overflow of NA in the absence of procaterol - $0.43 \pm 0.04\%$, $0.37 \pm 0.05\%$, $0.42 \pm 0.05\%$ and $0.39 \pm 0.04\%$; renal NA content in procaterol group - 468 ± 37 , 481 ± 12 , 476 ± 18 and 423 ± 22 pmol g^{-1} . For other experimental conditions, refer to Figure 1. (b) and (d) Schild plots of ICI 118,551 at prejunctional β -adrenoceptors using (-)-isoprenaline (b) and procaterol (d) as agonists. The slopes of the regression lines in (-)-isoprenaline experiments (1.19) and procaterol experiments (0.95) were not significantly different from unity. Therefore, presented above are the lines constrained to a slope of 1, which were best fitted to the points. From these lines, the pK_b values were determined to be 9.20 and 9.35.

of a small tubular population of the β_2 -subtype (Figure 6). Surgical denervation had little effect on this pattern (data not shown). β_2 -Adrenoceptors also were present in the outer medulla, where they are most probably associated with collecting tubules (Figure 7). Intraparenchymal branches of the renal artery displayed silver grains in the adventitia and adventitial-medial border; these binding sites were displaced by ICI 118,551 and thus represent β_2 -adrenoceptors (Figure 8). No silver grain was detectable in the arterial muscle layer (Figure 8). Importantly, surgical denervation resulted in a complete disappearance of this population, suggesting a pre-junctional localization of these binding sites (Figure 8). No specific β_1 -adrenoceptor binding was noticeable within sections of intraparenchymal branches of the renal artery (data not shown).

Discussion

β -Adrenoceptor activation results in facilitation of S-I NA overflow from sympathetic varicosities. However, some basic aspects of these receptors have not been delineated properly. It is not known exactly which subtype of β -adrenoceptors mediates the facilitatory response. Similarly, the anatomical location of these receptors within peripheral organs has not been examined in detail. The present investigation was undertaken to define these aspects by use of two different approaches: pharmacological and anatomical. Pharmacologically, the receptors were characterized using classical pharmacological methods; examination of agonist potency ratios and pA_2 or pK_b determinations. Anatomically, the receptors were characterized and their anatomical location was defined using radio-ligand binding and autoradiography techniques.

Although the classical pharmacological methods (potency ratio determination, pA_2 or pK_b determination) have been used extensively to characterize postjunctional β -adrenoceptors, these methods have not been applied to date to characterize prejunctional β -adrenoceptors because of various complications in achieving proper experimental conditions. One prerequisite for the correct characterization of any receptor is that the concentration of an agonist in the vicinity of the receptors should be estimated as accurately as possible (Furchgott, 1972). Also, the receptor system should be functionally isolated and the receptor-mediated response should be minimally influenced by other endogenous or exogenous factors (Furchgott, 1972). Hence, with regard to prejunctional β -adrenoceptors, the final determination of the potency ratio or pK_b value may be influenced by (1) a co-transmitter released with NA (Bartfai *et al.*, 1988) which may influence the facilitatory response; (2) simultaneous activation of other

Figure 4 (a) and (c) Concentration-facilitatory response curves to (-)-isoprenaline (a) and procaterol (c) in the absence (●) and presence of 3 μM (○), 10 μM (■) and 30 μM (□) metoprolol, $n = 4-6$. Data are presented as mean $\pm$ s.e.mean. The following values are for control, metoprolol (3 μM), metoprolol (10 μM) and metoprolol (30 μM) groups, respectively: fractional overflow of noradrenaline (NA) in the absence of (-)-isoprenaline – $0.57 \pm 0.09\%$, $0.52 \pm 0.07\%$, $0.58 \pm 0.07\%$ and $0.56 \pm 0.10\%$; renal NA content in (-)-isoprenaline group – 349 ± 49 , 327 ± 21 , 332 ± 29 and 326 ± 34 pmol g^{-1} ; fractional overflow of NA in the absence of procaterol – $0.46 \pm 0.04\%$, $0.42 \pm 0.04\%$, $0.43 \pm 0.03\%$ and $0.51 \pm 0.11\%$; renal NA content in procaterol group – 346 ± 18 , 333 ± 31 , 382 ± 37 and 437 ± 88 pmol g^{-1} . For other experimental conditions, refer to Figure 1. (b) and (d) Schild plots of metoprolol at prejunctional β -adrenoceptors using (-)-isoprenaline (b) and procaterol (d) as agonists. The slopes of the regression lines in (-)-isoprenaline experiments (1.08) and procaterol experiments (0.97) were not significantly different from unity. Therefore, presented above are the lines constrained to a slope of 1, which were best fitted to the points. From these lines, the pK_b values were determined to be 6.25 and 6.18.

Table 2 Effects of β -adrenoceptor antagonists on agonist-concentration-facilitatory response curves

Agonist	Antagonist	Schild plot slope ^a (95% confid. limits)	pK_b ^b	Antagonist conc.	Shift of agonist curve ^c	Agonist max. response ^d
(-)-Isoprenaline	ICI 118,551	1.19 (0.3–2.0)	9.2	0		114 ± 33%
				3 nM	6	109 ± 18%
				10 nM	20	123 ± 13%
				30 nM	83	117 ± 17%
Procaterol	ICI 118,551	0.95 (0.89–1.00)	9.35	0		101 ± 8%
				3 nM	8	128 ± 15%
				10 nM	25	103 ± 19%
				30 nM	72	108 ± 8%
(-)-Isoprenaline	Metoprolol	1.08 (0.48–1.67)	6.25	0		87 ± 11%
				3 μ M	5	76 ± 9%
				10 μ M	17	101 ± 9%
				30 μ M	50	86 ± 9%
Procaterol	Metoprolol	0.97 (0.93–1.00)	6.18	0		97 ± 21%
				3 μ M	5	108 ± 5%
				10 μ M	13	89 ± 15%
				30 μ M	38	79 ± 17%

^aSchild plot slope of the line obtained by best fit of the data points in Figures 3b, 3d, 4b or 4d along with the 95% confidence limits for that line.

^bX-intercept of Schild Plots shown in Figures 3b, 3d, 4b or 4d where a line constrained to a slope of 1 was best-fitted to the data points.

^cDextral parallel shifts (EC_{50} of the agonist in presence of [antagonist]/ EC_{50} of the agonist in absence of antagonist). Data are shown in Figures 3a, 3c, 4a and 4c.

^dFacilitation of stimulus-induced fractional neurotransmitter overflow expressed as a percentage of the overflow in the absence of agonist. The antagonists alone had no effect on overflow at the concentrations studied.

Table 3 Effect of surgical sympathetic denervation on noradrenaline (NA) content, β_1 -adrenoceptors and β_2 -adrenoceptors in the rat kidney

	NA content (ng g ⁻¹ tissue)	β_1 -Adrenoceptors (fmol mg ⁻¹ tissue)	β_2 -Adrenoceptors (fmol mg ⁻¹ tissue)
Control (sham-operated)	34 ± 5	68 ± 10	40 ± 11
Denervation	4 ± 2*	53 ± 14	8 ± 7*

*Significantly different as compared to the control condition, $P < 0.01$.

prejunctional receptors on sympathetic nerve terminals (Rump, 1987); (3) active removal of NA or β -adrenoceptor agents, and (4) endogenous factors generated in the organ, especially angiotensin II and prostaglandins in the present case (Boke & Malik, 1983; Malik, 1988). Therefore, care was taken in the present study to minimize the influence of these factors. First, it has been suggested that ATP plays the role of a sympathetic co-transmitter in the rat kidney which, however, has no effect on S-I overflow of NA (Schwartz & Malik, 1989). Second, only prejunctional α -adrenoceptors are tonically active under the experimental conditions of the present study and influence of prejunctional α -adrenoceptors on sympathetic neurotransmission was minimized in the present study by using phentolamine. Third, active uptake of NA or β -adrenoceptor agents was prevented by inhibiting neuronal and extraneuronal uptake. Finally, synthesis of renal angiotensin II and prostaglandins was inhibited with captopril and indomethacin to eliminate their potential influence on β -adrenoceptor-mediated facilitation.

Another important point in the use of pharmacological methods for characterizing prejunctional β -adrenoceptors is the choice of β -adrenoceptor agents. O'Donnell & Wanstall (1979) pointed out that the pA_2 values obtained with selective receptor agonists and antagonists provide the best basis for receptor differentiation. They demonstrated that the pA_2 values for a selective antagonist may differ against agonists of different selectivities in tissues containing more than one receptor subtype. Hence, β -adrenoceptor agonists and antagonists with different selectivities for β -adrenoceptor subtypes were used in the present investigation to differentiate between the β_1 -, β_2 - and β_3 -subtypes as prejunctional β -adrenoceptors.

One strategy which can provide evidence for the involvement of a particular receptor subtype in a functional response is examination of the rank order of potency and potency ratios of various β -adrenoceptor agonists (Kenakin, 1984). In the present investigation, all the agonists, except dobutamine, acted as full agonists at prejunctional β -adrenoceptors in the rat kidney. The rank order of potency of β -adrenoceptor agonists was, (-)-isoprenaline > procaterol > salbutamol > adrenaline > (+)-isoprenaline, which is indicative of the β_2 -subtype since at the β_1 -subtype, adrenaline is more potent than salbutamol and procaterol. This rank order of potency also confirmed the stereoselectivity of prejunctional β -adrenoceptors as (-)-isoprenaline was observed to be more potent at prejunctional β -adrenoceptors than (+)-isoprenaline. Another important observation in the present investigation was that the potency ratios of the β -adrenoceptor agonists are similar to their reported ratios at β_2 -adrenoceptors, and differ significantly from their reported ratios at β_1 -adrenoceptors (see Table 1). Thus, these results suggest that prejunctional β -adrenoceptors are of the β_2 -subtype.

In contrast to the other β -adrenoceptor agonists used, the preferential β_1 -adrenoceptor agonist, dobutamine, did not produce any facilitation of S-I overflow of NA. This may be due to the presence of the β_2 -subtype as prejunctional β -adrenoceptors. However, two alternative explanations should be considered. One possibility is that the concentrations of dobutamine used in the present study may not be sufficient to activate prejunctional β -adrenoceptors. Dobutamine is 200 to 1000 fold less potent than isoprenaline at the β -adrenoceptor subtypes (Wagner & Schumann, 1979; Kenakin, 1981). Since

Figure 5 (a) Total (○), specific (●) and nonspecific (□) [¹²⁵I]-cyanopindolol ([¹²⁵I]-CYP) binding to sections of the rat kidney, $n = 10$. Data are presented as mean $\pm$ s.e.mean. The [¹²⁵I]-CYP binding was concentration-dependent. (b) Displacement curves of [¹²⁵I]-CYP binding by ICI 89,406 (◇) and ICI 118,551 (□), $n = 10$. The data are presented as mean $\pm$ s.e.mean.

the EC₅₀ value for (–)-isoprenaline at prejunctional β -adrenoceptors was observed to be 1–2 nM, dobutamine should produce facilitation in the concentration range of 1 to 10 μ M which was examined in the present study. Thus, the concentrations of dobutamine studied were appropriate. Second, dobutamine can activate α -adrenoceptors in addition to its action at β -adrenoceptors (Ruffolo *et al.*, 1981). Although experiments in the present study were performed in the presence of 10 μ M phentolamine, it is possible that dobutamine may have overcome the α -adrenoceptor blockade, especially at higher concentrations, and may have produced inhibition of sympathetic neurotransmission by activating prejunctional α -adrenoceptors. This inhibition may have counterbalanced the facilitatory influence of dobutamine by β -adrenoceptor stimulation. This possibility is unlikely since no vasoconstriction (indicative of α -adrenoceptor activation) was observed in the presence of dobutamine. Thus, the results with dobutamine support the presence of the β_2 -adrenoceptors as prejunctional β -adrenoceptors.

Another strategy which was used in the present study to characterize the prejunctional β -adrenoceptor was the determination of antagonist pA₂ or pK_b values. Propranolol, the β -adrenoceptor antagonist, has high affinity (in nM range) for the β_1 - and β_2 -subtypes, but low affinity (in μ M range) for β_3 -adrenoceptors (Bond & Clark, 1988). Hence, this property of propranolol was taken advantage of to examine whether β_3 -adrenoceptors are responsible for the facilitatory response. Theoretically, if β_3 -adrenoceptors contributed significantly to

Figure 6 Autoradiography of [¹²⁵I]-cyanopindolol ([¹²⁵I]-CYP) binding in the rat renal cortex. (a) Dark-field picture of a section incubated in the presence of ICI 118,551 to block binding to β_2 -adrenoceptors. β_1 -Adrenoceptors are present mainly within glomeruli. (b) Bright-field picture of the same field as shown in (a) to verify anatomical details. (c) Dark-field picture of a section incubated in the presence of ICI 89,406 to block binding to β_1 -adrenoceptors. β_2 -Adrenoceptors are associated mainly with renal tubules. G = glomeruli, T = tubules. (Magnification: $\times 125$, calibration bar = 50 μ m).

the facilitation of sympathetic neurotransmission in the rat kidney, 30 nM propranolol should produce a shift of the concentration-response curve to (–)-isoprenaline, the nonselective β -adrenoceptor agonist, which is less than the shift expected for antagonism at β_1 - or β_2 -adrenoceptors. However, a parallel, dextral shift of the (–)-isoprenaline curve was observed in the presence of 30 nM propranolol and the pA₂ of propranolol at prejunctional β -adrenoceptors was determined to be 9.20. This pA₂ value is similar to the pA₂ values of propranolol in guinea-pig atria (β_1 -subtype) and guinea-pig trachea (β_2 -subtype) which are 8.6 and 8.4, respectively, (Bertholet *et al.*, 1981), but significantly different from its pA₂ value at β_3 -adrenoceptors which is 6.6 (Wilson *et al.*, 1984).

Figure 7 Autoradiography of [125 I]-cyanopindolol ([125 I]-CYP) binding in the rat renal outer medulla. (a) Dark-field picture of a section incubated without displacing agents. (b) Bright-field picture of the same field as shown in (a) to verify microanatomical details. (c) Dark-field picture of a section incubated in the presence of ICI 118,551 to block binding to β_2 -adrenoceptors. Silver grains are remarkably reduced (magnification: $\times 125$, calibration bar = 50 μ m).

These results imply that the β_3 -adrenoceptor subtype does not significantly contribute to the facilitatory response in the rat kidney.

To differentiate between the β_1 - and β_2 -subtypes as prejunctional β -adrenoceptors, the pK_b values for ICI 118,551 and metoprolol at prejunctional β -adrenoceptors also were determined. ICI 118,551 exhibits 100 to 300 fold selectivity for β_2 -adrenoceptors over β_1 -adrenoceptors (Bilski *et al.*, 1983; Lemoine *et al.*, 1985). It has been suggested that ICI 118,551 may produce nonspecific inhibitory effects at 1 μ M or higher concentrations (Bilski *et al.*, 1983). However, no nonspecific effect of ICI 118,551 was observed at the concentrations used. Importantly, the slopes of the Schild regression lines for ICI 118,551 obtained using (–)-isoprenaline and procaterol, the selective β_2 -adrenoceptor agonist, were observed

Figure 8 Autoradiography of [125 I]-cyanopindolol ([125 I]-CYP) binding in the intraparenchymal branch of the renal artery. (a) Dark-field picture of a section incubated in the presence of ICI 89,406 to block binding to β_1 -adrenoceptors. Silver grains representing β_2 -adrenoceptors are located in the adventitia and adventitial-medial border. (b) Bright-field picture of the same field as shown in (a) to verify microanatomical details. (c) Dark-field picture of a section incubated in the presence of (–)-propranolol to display nonspecific binding. (d) Dark-field picture of a section from a surgically denervated animal. The density of silver grains is remarkably reduced. L = lumen; Arrows indicate the adventitial-medial border. (Magnification: $\times 160$, calibration bar = 50 μ m).

not to be different from unity. Furthermore, the pK_b values for ICI 118,551 against (–)-isoprenaline and procaterol (9.20 and 9.35) were only 0.15 log unit apart. This demonstration of the same pK_b values and Schild regressions of unity for ICI 118,551 against agonists of differential selectivity is indicative that a homogeneous population of β -adrenoceptors is mediating the facilitation (O'Donnell & Wanstall, 1979). To determine exactly which subtype was involved in the facilitation, we compared the pK_b of ICI 118,551 at prejunctional

tional β -adrenoceptors with its reported pA_2 at the classical β -adrenoceptor subtypes. The pA_2 of ICI 118,551 in guinea-pig atria (β_1 -adrenoceptors) has been reported to be 6.96 (O'Donnell & Wanstall, 1980) and 7.17 (Bilski *et al.*, 1983). Alternatively, the pA_2 of ICI 118,551 in guinea-pig trachea (β_2 -adrenoceptors) has been reported to be 8.69 (O'Donnell & Wanstall, 1980), while at β_2 -adrenoceptors, it is 5.49 and 5.33 (Hollenga & Zaagsma, 1989). Thus, the pA_2 of ICI 118,551 at prejunctional β -adrenoceptors is similar to its pA_2 at β_2 -adrenoceptors, implying that a homogeneous population of β_2 -adrenoceptors may mediate the facilitation. However, a possibility of a small contribution by β_1 -adrenoceptors cannot be eliminated on the basis of these results, as a narrow concentration-range of ICI 118,551 was used in the present study within which a β_1 -adrenoceptor contribution may not have been apparent (Lemoine & Kaumann, 1983). This possibility was evaluated by determining the pK_b values of the selective β_1 -adrenoceptor antagonist, metoprolol.

Metoprolol has approximately 30 fold selectivity for β_1 -adrenoceptors as compared to β_2 -adrenoceptors (Toda *et al.*, 1978; Wahlund *et al.*, 1990). It has been demonstrated that metoprolol may exhibit a local anaesthetic-like effect at 1 μ M or higher concentrations (Doggrell, 1988). However, metoprolol did not affect sympathetic neurotransmission at the concentrations used implying that it did not produce any nonspecific effect. Similar to ICI 118,551, the slopes of the Schild regression lines for metoprolol were not significantly different from unity. Furthermore, the pK_b values for metoprolol against (–)-isoprenaline and procaterol at prejunctional β -adrenoceptors were 6.25 and 6.18, respectively, suggesting that its antagonism was at a single site. These values are similar to the pA_2 of metoprolol in guinea-pig trachea (representing its antagonism at β_2 -adrenoceptors) which are 6.06 (Harms, 1976) and 6.25 (Toda *et al.*, 1978), but different from its pA_2 in guinea-pig atria, representing its antagonism at β_1 -adrenoceptors, which are 7.43 (Harms, 1976) and 7.93 (Toda *et al.*, 1978). Together these results refute the involvement of β_1 -adrenoceptors in facilitation of S-I overflow of NA in the rat kidney and support the notion of a homogeneous population of β_2 -adrenoceptors mediating the response. The next question is: where are these receptors located?

Although traditionally it is believed that prejunctional β -adrenoceptors are located on sympathetic nerve terminals, very sparse anatomical evidence has been obtained to support this concept (Sharma & Banerjee, 1978; Dahlof *et al.*, 1986). Theoretically, if these receptors are located prejunctionally, then destruction of the sympathetic nerve terminals containing these receptors should result in loss of the receptors. We utilized this theoretical concept to examine the location (prejunctional or postjunctional) of 'prejunctional' β -adrenoceptors in the rat kidney using radio-ligand binding and autoradiography combined with surgical denervation.

The binding of [125 I]-CYP in the rat kidney slices was saturable and of high affinity. Furthermore, the affinity of [125 I]-CYP for renal β -adrenoceptors observed in the present investigation is similar to that reported in previous studies with the same radio-ligand in the rat kidney (Munzel *et al.*, 1984; Summers *et al.*, 1985). The binding studies revealed that of the total β -adrenoceptor population in the rat kidney, β_1 -adrenoceptors were 63% while β_2 -adrenoceptors were 37%. Other investigators also have reported similar values for renal β -adrenoceptor subtypes (Snively *et al.*, 1982; Summers *et al.*, 1985). However, a more important observation was that surgical denervation, accompanied by more than 90% reduction in renal NA content, resulted in 80% reduction in the β_2 -adrenoceptors. Disappearance of the β_2 -sites upon surgical denervation suggests a prejunctional location for these receptors. This result is in strong agreement with the results obtained by pharmacological methods which also demonstrated that prejunctional β -adrenoceptors are of the β_2 -subtype. In contrast to β_2 -adrenoceptors, the β_1 -adrenoceptor population was not significantly affected by

surgical denervation. This suggests that β_1 -adrenoceptors are not present on sympathetic nerve terminals. This suggestion is supported by the pharmacological demonstration that β_1 -adrenoceptors are not involved in facilitation of S-I overflow of NA in the rat kidney. The results obtained in the present study are at variance with those by Fortin & Sundaresan (1987) and Woodcock *et al.* (1985) who failed to observe any effect of sympathectomy on β -adrenoceptor population in the rat kidney. However, Fortin & Sundaresan (1989) used only the renal cortex to examine the β -adrenoceptor population in that study, in contrast to the whole kidney preparation used in the present study, and this may account for the different results. On the other hand, Woodcock *et al.* (1985) failed to observe the effect of chemical sympathectomy on β -adrenoceptors in the whole kidney. The reason for the discrepancy between this and our results is not known.

The prejunctional localization of prejunctional β -adrenoceptors also was confirmed in the present investigation by use of autoradiography. Autoradiographic analysis of renal β -adrenoceptors demonstrated that β -adrenoceptors were localized in cortex and outer medulla. Cortical β -adrenoceptors, associated with glomeruli and tubules, were mainly β_1 in nature with a small population of β_2 -adrenoceptors present on the tubules. In contrast, collecting tubules present in outer medulla mainly contained β_2 -adrenoceptors. These localizations of renal β -adrenoceptors are in general agreement with other reports (Summers & Kuhar, 1983; Summers *et al.*, 1985; Healy *et al.*, 1985). Surgical denervation did not alter the density of β -adrenoceptor subtypes present at cortical and outer medullary locations, implying that these receptors are not associated with renal sympathetic nerves innervating these structures (Barajas & Powers, 1990). An important observation was the presence of β_2 -adrenoceptors on the adventitia and adventitial-medial border of the intraparenchymal branches of the renal artery. β_2 -Adrenoceptors on the renal vasculature also have been reported by Summers *et al.* (1985). It has been demonstrated previously that the intraparenchymal branches are innervated by sympathetic nerves (Barajas & Powers, 1990). Since the adventitia and adventitial-medial border of an artery represent the vascular zones where sympathetic nerve plexus ends (cf. Amenta *et al.*, 1991), β_2 -adrenoceptors associated with the intraparenchymal branches of the renal artery may represent prejunctional β_2 -adrenoceptors located on renal sympathetic nerve terminals. The conclusive evidence for this hypothesis was obtained when it was observed that this population of β_2 -adrenoceptors disappeared following destruction of the nerve terminals by surgical denervation. Thus, these results provide the direct anatomical evidence for the presence of β_2 -adrenoceptors on the renal sympathetic nerve terminals associated with renal vasculature. Furthermore, these results suggest that prejunctional β -adrenoceptors in the rat kidney may not affect tubular function directly. Instead, they may influence renal function by altering renal hemodynamics.

In summary, the present study demonstrates that renal prejunctional β -adrenoceptors are of the β_2 -subtype in nature. Anatomical evidence was obtained suggesting that these receptors are located on sympathetic nerve terminals associated with the renal vasculature rather than renal tubules. Thus, prejunctional β_2 -adrenoceptors in the rat kidney may influence renal function by altering sympathetic neurotransmission at the renal vasculature level.

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