Biochemical Pharmacology, Vol. 31, No. 4, pp. 583–587, 1982.
Printed in Great Britain.

0006–2952/82/040583-05 $03.00/0
Pergamon Press Ltd.

LOCALISATION OF [^{3}H]CLONIDINE BINDING TO MEMBRANES FROM GUINEA PIG RENAL TUBULES

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(*Received* 1 *May* 1981; *accepted* 17 *August* 1981)

Abstract—The selective radioligand [^{3}H]clonidine has been used to localise α_2 adrenoceptors in guinea pig kidney. Chemical sympathectomy with 6-hydroxydopamine produced no significant change in the number of sites labeled by [^{3}H]clonidine indicating that the majority of binding sites were not located on sympathetic nerve terminals. Binding was enhanced in membranes prepared from renal tubules and considerably reduced in preparations from glomeruli. Subcellular fractions of renal cortex revealed that binding was to plasma membranes and that the greatest binding capacity was present in the fraction rich in basal lateral membranes. It is concluded that the major concentration of renal α_2 adrenoceptors are present on renal tubules and that they may be localised to a particular pole of the renal tubule cell.

[^{3}H] Clonidine has been used as a radioligand to study α_2 adrenoceptors in membranes prepared from the kidneys of guinea pig [1] and rat [2]. Within the guinea pig kidney the α_2 adrenoceptors are highly localised to membranes prepared from the renal cortex with membranes from medulla and papilla having much lower concentrations [3, 4]. Recent autoradiographic studies have confirmed these findings and extended them to show that the α_2 adrenoceptors are located on the proximal convoluted tubules [5]. The results are consistent with the presence of α adrenoceptors on proximal convoluted tubules which mediate sodium reabsorption [6].

In the experiments described here [^{3}H]clonidine binding within the guinea pig renal cortex has been localised using the techniques of chemical sympathectomy with 6-hydroxydopamine, preparation of membranes from glomeruli and tubules and subcellular fractionation. A preliminary account of some of this work has been previously described [7].

MATERIALS AND METHODS

Chemical sympathectomy. Male guinea pigs (500–800 g) were given 6-hydroxydopamine (150 mg/kg i.p.). Animals were killed and the kidneys removed 5, 7 or 11 days after injection. The extent of sympathectomy was estimated by measurement of the catecholamine content of a sample of tissue by radioenzymatic assay [9]. Only kidneys in which the noradrenaline content was less than 5 per cent of the controls were used in this study.

Preparation of glomeruli and tubules. Kidneys were removed from male guinea pigs and placed on ice. Renal cortex was dissected from medulla and papilla, minced with a razor blade, forced through a 212 micron nylon mesh and suspended in isotonic Tris 50 mM/sucrose 8%: pH 7.6 at 4°. This procedure disrupted the tubular structures and freed the glomeruli which were then separated from the debris by a combination of differential centrifugation and filtration. The glomerular suspension obtained after the initial sieving was centrifuged (1000 *g* for 1 min), the supernatant removed and the pellet was resuspended in fresh buffer. This procedure was repeated a further five times before passing the suspension through an 85 micron nylon mesh. The majority of the remaining tubule fragments passed through the mesh leaving the glomeruli (dia ~ 120 microns) on the surface.

Tubules were prepared from renal cortex incubation in Krebs–Henseleit solution containing 0.05% collagenase (Sigma type II) for 30 min at 37° with vigorous shaking (120 shakes/min) [10]. After digestion the slices were disrupted by repeated passage into a Pasteur pipette. The suspension was then passed gently through a 212 micron nylon mesh to complete dispersion. After three washes in collagenase free Krebs the suspension was layered onto a discontinuous sucrose gradient (75, 58 and 50%) and spun at 5000 *g* for 35 min. Tubules were collected from the interface (50:58) and purity checked by microscopic examination. Membranes from both tubules and glomeruli were prepared by homogenising the structures in Tris buffer (50 mM, pH 7.6 at 4° and 25°) as previously described [4].

Subcellular fractionation. Subcellular fractionation was carried out, using a homogenate of guinea pig renal cortex [11]. Briefly, renal cortex was minced and homogenised in 10 vol. of 8% sucrose (pH 7.0) using 10 strokes of a Dounce homogeniser with a tight fitting pestle. A sample of this homogenate was retained (H1). The remainder was centrifuged at 1000 *g* for 10 min, the supernatant saved and the pellet resuspended in 2.5 vol. of 8% sucrose. After rehomogenisation and centrifugation the supernatant was combined with that from the first spin and the pellet (P1) retained. The supernatant was spun at 9000 *g* for 10 min. The lower pellet (P2) was retained whereas the soft upper portion was combined with the supernatant and centrifuged at 47,000 *g* for 20 min. The clear supernatant from this spin was discarded. The upper lighter portion of the

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pellet was used to prepare fractions enriched in luminal (P4) and basal–lateral (P5) membranes by selective precipitation and the lower pellet was retained (P3A). The fractions H1, P1, P2, P3A, P4 and P5, were washed at least twice by suspension in Tris–HCl buffer (pH 7.6 at 4°) and centrifuged at 49,000 *g* for 10 min. Finally the fractions were suspended in Tris–HCl buffer pH 7.6 at 25° (protein concentration 1–3 mg/ml).

In some experiments enzyme marker assays for Na^+/K^+ ATPase [12] and alkaline phosphatase [13] were conducted to assess the degree of enrichment of each membrane fraction.

Chemicals. Drugs used in the present experiments were; [^{3}H]clonidine, 22–26 Ci/mmole (NEN), 6-hydroxydopamine hydrobromide and collagenase (type II) (Sigma); phentolamine hydrochloride (Ciba). All other reagents were 'analar' grade chemicals.

RESULTS

The effects of 6-hydroxydopamine pretreatment

The effect of 6 hydroxydopamine pretreatment on [^{3}H]clonidine binding to membranes prepared from the renal cortex is seen in Fig. 1. There was a reduction in the apparent dissociation constant (K_d) from approximately 8 to 4 nM which was dependent on the time after denervation, the change being maximal after 11 days. In contrast, there was little change in the maximal number of binding sites (B_{max}) from 17 pmoles/g wet wt of tissue in all cases. The Hill coefficient (nH) did not significantly differ from unity in all experiments, indicating no change in cooperativity with 6-hydroxydopamine administration. The results for all experiments are summarised in Table 1. The lack of change in the density of binding sites indicates that the bulk of the renal α_2 adrenoceptors labeled by [^{3}H]clonidine are located other than on sympathetic nerve terminals. However, since the standard deviation about the mean density of binding sites was ± 22.6 per cent, it is possible that small changes produced by denervation would not be seen against a much larger population of sites not associated with nerves.

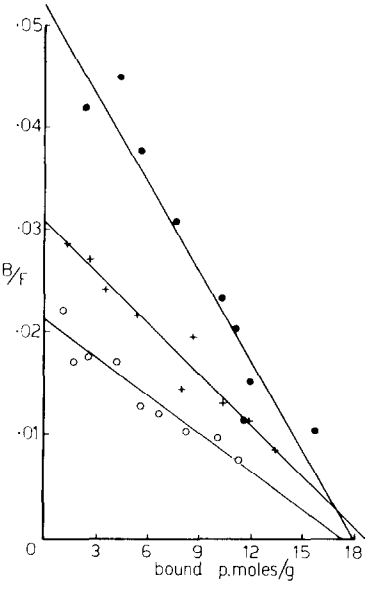

Fig. 1. The effect of chemical sympathectomy with 6-hydroxydopamine on [^{3}H]clonidine binding to membranes from guinea pig renal cortex. Scatchard plots are shown for control (○) and animals sympathectomised 7d (+) or 14d (●) previously.

[^{3}H]*Clonidine binding to glomeruli and tubules*

In membranes prepared from the renal cortex the K_d for [^{3}H]clonidine binding was 8.3 ± 0.3 nM with a B_{max} of 186 ± 25 fmoles/mg protein ($N = 4$). After sieving or collagenase digestion, the apparent affinity of [^{3}H]clonidine binding increased to 3 nM while the B_{max} was reduced to ⩾ 95 fmoles/mg protein (Table 2). Since the preparation of glomeruli or tubules followed sieving or collagenase digestion the controls for glomeruli were the suspension obtained after sieving and for tubules, the suspension obtained after collagenase digestion and disruption with the pasteur pipette.

[^{3}H]Clonidine binding was not localised to glomeruli since membranes prepared from these structures showed a reduced B_{max} (Fig. 2). In two of the four experiments the B_{max} was 47 fmoles/mg protein or about half that in controls. In the remaining two experiments no specific binding was observed. The suspension that remained after sieving (the glomeruli free preparation) showed a slight increase in B_{max} to 134.4 fmoles/mg protein ($n = 4$) compared to control. Since glomeruli form only 1–2 per cent of the mass of the renal cortex and have a lower binding capacity than the starting material it is clear that they can be responsible for only a small (<1%)

Table 1. Effect of 6-hydroxydopamine pretreatment on [^{3}H]clonidine binding constants in membranes prepared from guinea-pig renal cortex

Time	B_{max} (pmol g^{-1} wet wt)	K_d (nM)	nH	n
Control	16.4 ± 1.1	7.4 ± 0.9	0.99 ± 0.01	8
5 days	17.3 ± 3.3	6.3 ± 0.7	0.94 ± 0.02	3
7 days	17.7 ± 1.7	4.5 ± 0.05	1.0 ± 0.02	8
11 days	17.4 ± 0.7	3.6 ± 0.1	1.02 ± 0.05	3

Table 2. [^{3}H]Clonidine binding constants in membranes from guinea pig renal tubules and glomeruli

		B_{max} (fmol mg^{-1}protein)	K_d (nM)	nH
Tubules				
n = 4	Control	95.5 ± 9.7	3.3 ± 0.5	1.00 ± 0.01
	Purified tubules	167.5 ± 13	4.0 ± 0.6	1.00 + 0.01
Glomeruli	Control	94.8 ± 16	3.3 ± 0.6	1.0 ± 0.01
n = 4	Purified Glomeruli	46.5	4.88	0.86
	Glomeruli free preparation	134.4 ± 24	4.20 ± 1.4	0.97 ± 0.03

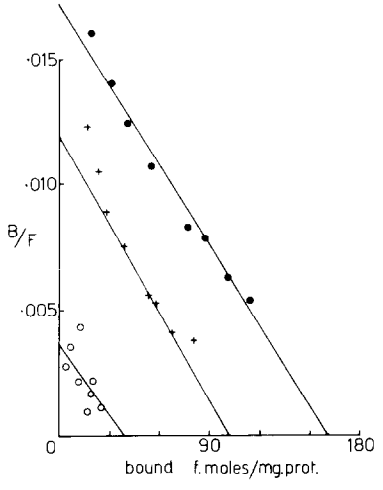

Fig. 2. Scatchard plot of binding of [^{3}H]clonidine to membranes prepared from glomeruli (○), tubules (●) or collagenase treated renal cortex (+).

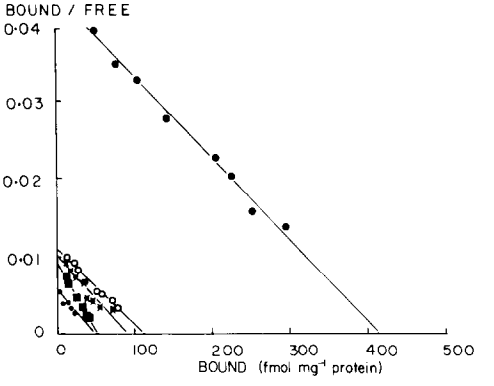

Fig. 3. Scatchard plots of [^{3}H]clonidine binding to membrane fractions prepared from guinea pig renal cortex: (○) starting homogenate H1, (●) P1 — cell debris, (✖) P3A — plasma membrane fraction, (■) P4 — luminal membrane enriched, (●) P5 — basal lateral membrane enriched.

fraction of the binding seen in membranes prepared from renal cortex. The dissociation constants for [^{3}H]clonidine binding in control, glomerular and control minus glomerular, fractions were similar (Table 2).

Membranes prepared from renal tubules collected at the 50/58% sucrose interface showed enhanced binding, having a B_{max} of 167.5 ± 13.0 fmoles/mg protein compared with 95.5 ± 9.7 fmoles/mg protein in controls (Fig. 2). Values for the K_d's in the two preparations were 3.3 ± 0.5 and 4.0 ± 0.6 nM (N.S.). In all cases Hill coefficients did not differ significantly from unity indicating no cooperativity in binding.

Table 3. [^{3}H]Clonidine binding constants in subcellular fractions from guinea pig renal cortex

Fraction	Bmax fmol/mg protein	K_d nM	nH	n
H1	134.1 ± 15	6.5 ± 0.6	1.00 ± 0.01	4
P1*	113.6	10.2	0.96	2
P2	28.2 ± 4.1	12.9 ± 3.3	0.97 ± 0.03	4
P3A	94.8 ± 15.8	5.6 ± 1.8	0.99 ± 0.03	4
P4	93.4 ± 17.6	3.2 ± 0.5	1.02 ± 0.01	4
P5	251.7 ± 43	4.3 ± 0.3	1.00 ± 0.01	4

* In 2 experiments the membranes prepared from the P1 fraction filtered too slowly for valid estimates to be made of the binding constants. The results from these experiments have been excluded.

Table 4. Protein content and total binding capacity of subcellular fractions prepared from guinea pig renal cortex

Fraction	Protein Content	Total binding	n
	mg	Capacity % total	
P1	79.1 ± 15.1	4.1 ± 2.1	3
P2	172 ± 41	8.2 ± 1.9	3
P3A	122 ± 17	23.5 ± 7.5	3
P4	24.5 ± 4.0	9.2 ± 3.7	3
P5	115.7 ± 3.4	54.9 ± 3.3	3

[^{3}H]Clonidine binding to membrane fractions from guinea pig renal cortex

Subcellular fractions were prepared from guinea pig renal cortex. Assay of marker enzymes, Na/K ATPase and alkaline phosphatase, revealed that there was a 2-fold enrichment of Na/K ATPase activity (antiluminal membrane marker) in P5 and a 4 fold enrichment of alkaline phosphatase activity (luminal membrane marker) in P4. Scatchard plots of the results of representative experiments are shown in Fig. 3. The K_d obtained in all fractions was similar but the B_{max} showed marked variation with greatest enhancement of [^{3}H]clonidine binding being obtained in the P5 fraction.

To determine what contribution [^{3}H]clonidine binding in each fraction contributes to that seen in whole kidney cortex, protein recovery was measured in 3 of the experiments, and the results are shown in Table 4. Some 66 ± 9 per cent of total binding capacity was recovered in the experiments. The total binding capacity of each fraction was calculated by multiplying the maximal density of binding sites by the protein content in the fraction. The greatest binding capacity was observed in the P5 fraction which contained more than 50 per cent of the total binding capacity of the original homogenate.

DISCUSSION

[^{3}H]Clonidine binding to α_2 adrenoceptors in guinea pig kidney is localised to the renal cortex [4]. Segments of the nephron which are largely confined to the cortex include glomeruli, and proximal and distal convoluted tubules. Since the bulk of the renal sympathetic innervation is to juxtaglomerular cells and clonidine selectively acts on prejunctional α_2 adrenoceptors [14] the possibility that a fraction of the [^{3}H]clonidine binding in kidney membranes is to prejunctional receptors was explored. Chemical sympathectomy with 6-hydroxydopamine failed to reduce the maximum number of binding sites at 5, 7 or 11 days, the only change being an increase in apparent affinity. The reasons for the increase in affinity are not clear. It is unlikely that endogenous noradrenaline remains bound to receptors during membrane preparation thereby lowering the affinity of binding since preincubation of membranes at 37° for 1 hr had little effect on the K_d and measurements of catecholamines in the final pellet using a sensitive high performance liquid chromatographic–electrochemical detector system failed to reveal detectable amounts (McPherson and Summers, unpublished results). It is more likely that long term removal of transmitter by 6-hydroxydopamine increases the proportion of α_2 adrenoceptors in the high affinity (α_{2H}) state [17] causing an increase in apparent affinity of binding. This effect is similar to that observed in other tissues [15, 16]. Since neural tissue forms only a small fraction of total tissue mass, the majority of α_2 adrenoceptors labeled by [^{3}H]clonidine are located postjunctionally in the kidney as in other tissues [18]. This conclusion was supported by the results of these experiments in which particular structures were isolated from renal cortex.

The techniques involved in the preparation of glomeruli or tubules produced some changes to the binding parameters. After either sieving or collagenase digestion the apparent K_d decreased as did the B_{max}. The controls in these studies were therefore sieved (glomeruli) or collagenase treated (tubules) suspensions of renal cortex. The α_2 adrenoceptors were not localised to glomeruli since membranes prepared from these structures, which form only 1–2 per cent of the mass of the cortex, showed a reduced density of binding sites whereas membranes from renal tubules showed almost double the density of binding sites compared to controls. These results are in agreement with those of Young and Kuhar [5] in which α_2 adrenoceptors in guinea pig kidney labeled by [^{3}H]clonidine and visualised by autoradiography were shown to be localised to the proximal convoluted tubule with little labeling to glomeruli.

The preparation of membrane fractions from guinea pig renal cortex indicated that binding was largely confined to fractions rich in plasma membranes. The membrane markers in fraction P4 and P5 indicate that these fractions were enriched in luminal and antiluminal membranes respectively. The only fraction to display marked enrichment of binding was P5 which could indicate that the α_2 adrenoceptors are localised to antiluminal membranes or at lease to membranes that come down with this fraction.

The results reported here provide evidence that α_2 adrenoceptors in guinea pig kidney are located in plasma membranes derived from renal tubules. Few if any of the receptors are associated with glomeruli or sympathetic nerves. In dogs, renal infusion of noradrenaline increases reabsorption of sodium in the proximal tubule and the effect is independent of changes in renal haemodynamics. The effects of

clonidine are consistent with its partial agonist character. Low (agonist) doses produce a decreased excretion of sodium [19] or no change [20–22] whereas high (antagonist) doses have a natriuretic effect [20, 23, 24]. The evidence supports the concept of α adrenoceptors in renal proximal tubules which mediate sodium reabsorption.

In conclusion this study demonstrates that the bulk of α_2 adrenoceptors labeled by [^{3}H]clonidine in guinea pig kidney are located on renal tubules, not on glomeruli or sympathetic nerve endings. [^{3}H]Clonidine binding is markedly enriched in preparations rich in basal lateral membranes indicating that α_2 adrenoceptors may be further localised to a particular pole of the renal tubule cell.

Acknowledgements—The authors wish to acknowledge support by the National Health and Medical Research Council.

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