Benzodiazepine receptors along the nephron: [^{3}H]PK 11195 binding in rat tubules

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Binding of [^{3}H]PK 11195, an isoquinoline carboxamide derivative, was measured in microdissected tubule segments of rat nephron. High specific binding capacities (1.1–1.8 fmol·mm^{-1}) were found in the thick ascending limb of the Henle's loop and in the collecting tubule, whereas specific binding could not be detected in the proximal tubule. In the medullary collecting tubule, the association and dissociation rate constants at 4°C were $k_1 = 3.0 \times 10^6$ M^{-1}·min^{-1} and $k_{-1} = 0.021$ min^{-1}; the ratio $k_{-1}/k_1 = 7.0$ nM was in agreement with the estimated equilibrium dissociation constant ($K_d = 2.4$ nM). [^{3}H]PK 11195 binding sites from medullary ascending limb and medullary collecting tubule revealed the following sequence of specificity: PK 11195 = Ro 5-4864 ≫ clonazepam, indicating that tubule binding sites might be the peripheral benzodiazepine receptors of the rat kidney.

Nephron microdissection *PK 11195 binding* *Benzodiazepine*

1. INTRODUCTION

High-affinity receptors for benzodiazepines have been found in the brain and several other tissues including the kidney [1–4]. The neuronal receptors have been distinguished from those present on glial elements and peripheral tissues on the basis of their respective recognition patterns for a series of benzodiazepine analogues. Thus, clonazepam, a potent anxiolytic drug, binds with high affinity to brain receptors and with low affinity to peripheral receptors. Conversely, Ro5-4864, a benzodiazepine devoid of anxiolytic activity, exhibits low affinity for central receptors and high affinity for peripheral receptors [1–4]. Furthermore, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide, or PK 11195, a product chemically unrelated to the benzodiazepines, was at least potent as Ro5-4864 for displacing [^{3}H]Ro5-4864 in all organs tested [5,6].

The aim of this work was to determine the localization of peripheral benzodiazepine receptors along the rat nephron by measuring binding of [^{3}H]PK 11195 in discrete nephron segments obtained by microdissection. The results indicate that significant binding to receptors of high affinity occurs in the glomeruli, the thick ascending limbs of the Henle's loop and in the collecting tubules.

Abbreviations: PCT, proximal convoluted tubule; PR, pars recta; MAL, medullary thick ascending limb; CAL, cortical ascending limb; CCT, cortical collecting tubule; MCT, medullary collecting tubule

2. MATERIALS AND METHODS

2.1. Tubule microdissection

Animals used were male Wistar rats (200–250 g body wt) purchased from the Janvier Breeding Center (France). Tubule microdissection was performed as in [7]. The left kidney was perfused via the renal artery with 4 ml of a cold modified Hank's solution containing 0.25% collagenase (type I, Sigma), 0.1% bovine serum albumin and 1 mM $CaCl_2$. Thin pyramids cut along the corticopapillary axis were incubated in the same solution

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at 35°C for 15–20 min. After rinsing, the slices were microdissected by hand in ice-cold Hank's solution under stereomicroscopic observation. Tubules were photographed for length determination and kept at 4°C until assay.

2.2. [^{3}H]PK 11195 binding assay

Tubules from the same nephron segment (average total length, 1.0–2.0 mm) were incubated for 120 min at 4°C in a 2.5 μl droplet of medium containing 20 mM Tris–HCl (pH 7.4), 137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and various amounts of [^{3}H]PK 11195 (40 Ci/mmol, Amersham). Radioactivity bound to tubules was separated from free labelled ligand by rapid filtration of samples through Whatman GF/C glass filters. Filters were washed 5 times with 5 ml cold 20 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$. Radioactivity retained on the filter was counted for 50 min by liquid scintillation spectrometry. Non-specific binding was measured in the presence of 5 μM unlabelled PK 11195. Blanks incubated under identical conditions were run in each experiment.

The apparent dissociation constants for unlabelled PK 11195 and benzodiazepines were deduced from the determination of the dose-dependent inhibition of 5 nM [^{3}H]PK 11195 binding.

Control experiments indicated that: (i) keeping overnight tubules at 4°C did not alter specific and non-specific [^{3}H]PK 11195 binding and (ii) assaays performed for 120 min at 4°C or 20 min at 30°C gave the same values for maximal binding capacities, apparent dissociation constants and Hill coefficients.

3. RESULTS AND DISCUSSION

Fig.1 shows that total and non-specific binding of [^{3}H]PK 11195 to MAL and MCT was proportional to tubular length throughout the entire range tested, and the same observation also applied for CAL and CCT. On these grounds, results were expressed as fmol bound [^{3}H]PK 11195 per mm of tubule.

Specific binding of [^{3}H]PK 11195 to MCT was a process saturable with time (fig.2). At 4°C and for 7.7 nM ligand, about 60 min were needed to reach equilibrium, the half-time for binding being 15 min. Addition of a large excess of unlabelled

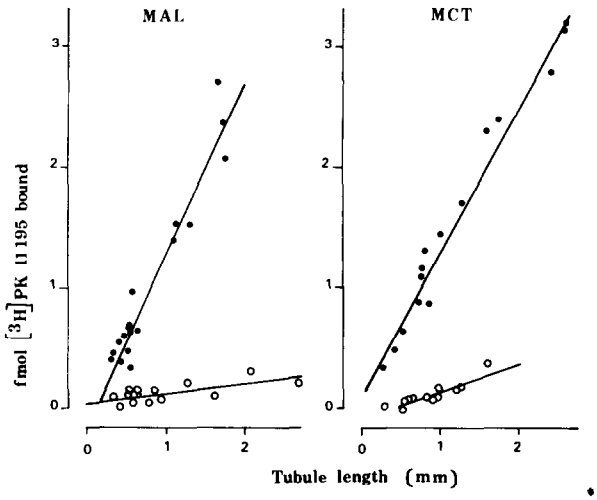

Fig.1. Effect of tubule length on total and non-specific [^{3}H]PK 11195 binding to medullary thick ascending limb (MAL) and medullary collecting tubule (MCT). Tubules were incubated with 20 nM [^{3}H]PK 11195 in the absence (total binding, ●—●) or presence (non-specific binding, ○—○) of 5 μM unlabelled PK 11195. The equations of the linear regression lines drawn on the graph are the following: MAL (●) $y = 1.43x - 0.11$ ($r = 0.964$, $P < 0.01$); MAL (○) $y = 0.09x + 0.04$ ($r = 0.774$, $P < 0.01$). MCT (●) $y = 1.20x + 0.12$ ($r = 0.987$, $P < 0.01$); MCT (○) $y = 0.23x - 0.09$ ($r = 0.888$, $P < 0.01$).

PK 11195 to pre-equilibrated tubules induced an almost dissociation of bound ligand.

The dose dependencies of total and non-specific [^{3}H]PK 11195 binding to MAL and MCT are illustrated in fig.3. The non-specific binding increased linearly and was negligible for concentrations lower than 5 nM. In both segments, the specific component of binding saturated in a ligand concentration range lower than 2 orders of magnitude. The transformation of the dose-dependent specific binding curves in the Scatchard coordinates generates curvilinear plots and in the Hill coordinates gives straight lines whose slopes are $n = 1.71$ and 1.56 for MAL and MCT, respectively. These data suggest the existence of a slight positive cooperativity in the interaction process between [^{3}H]PK 11195 and tubular binding sites. The apparent dissociation constants calculated from the x intercepts of the Hill plots are $K_d = 3.1$ and 2.4 nM for MAL and MCT, respectively. For MCT, this K_d value is in the same range as that calculated from the dissociation and association rate constants ($k_{-1}/k_1 = 7.0$ nM, see legend to fig.1).

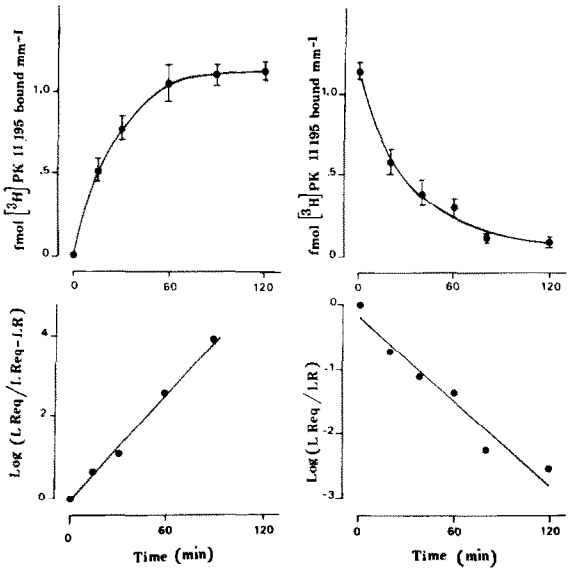

Fig.2. Kinetics of association and dissociation of [^{3}H]PK 11195 binding to medullary collecting tubules. MCT were incubated in the presence of 7.7 nM [^{3}H]PK 11195 and specific binding was measured as a function of incubation time at 4°C (left). Binding reversibility was induced by adding 5 μM unlabelled PK 11195 in the incubation medium of tubules preincubated for 120 min with 7.7 nM [^{3}H]PK 11195 and the remaining specific binding was measured as a function of time (right). Values are means ±SE of 4–5 determinations. The k_1 and k_{-1} association and dissociation rate constants were determined from the semilogarithmic plots shown in the lower part of the figure, assuming that the binding process corresponds to the reaction $L + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} LR$, in which L, R and LR are free ligand, free receptor and the ligand–receptor complex, respectively. The association and dissociation time courses are expressed by eqs1 and 2, respectively:

$$\log(LR\text{eq}/LR\text{eq} - LR) = (k_1 L + k_{-1})t \quad (1)$$

$$\log(LR/LR\text{eq}) = -k_{-1}t \quad (2)$$

in which LReq is the concentration of ligand–receptor complex at equilibrium. The equations of the linear regression lines drawn on the graph are the following: (left) $y = 0.044x - 0.056$ ($r = 0.999$, $P < 0.01$); (right) $y = -0.021x - 0.180$ ($r = 0.972$, $P < 0.01$). Computation gives $k_1 = 3.0 \times 10^6$ M^{-1}·min^{-1} and $k_{-1} = 0.021$ min^{-1}.

The detected [^{3}H]PK 11195 tubule binding sites exhibited a high degree of specificity (table 1). Ro5-4864, a benzodiazepine almost devoid of anxiolytic activity, was as effective as unlabelled PK 11195 in inhibiting [^{3}H]PK 11195 binding, whereas clonazepam, a potent anxiolytic benzodiazepine was found unable to interact with labelled binding sites. This ligand specificity is similar to that of the membrane-bound and solubilized benzodiazepine receptors from rat kidney [3–5]. Thus, it seems likely that tubule binding sites detected using the labelled ligand PK 11195, a product chemically unrelated to the benzodiazepines, might represent the peripheral benzodiazepine receptors of the rat kidney.

The distribution of [^{3}H]PK 11195 binding sites along the rat nephron is shown in table 2. High specific binding capacities were found in the glomeruli, MAL, CAL, CCT and MCT, whereas specific binding was not statistically different from zero in the PCT and PR. An effect of the collagenase pretreatment on the binding characteristics of various nephron segments is unlikely since this procedure did not alter the number of insulin receptors [8], the response of adenylate cyclase to various hormones [9] and the activity of ($Na^+ + K^+$)-ATPase [10,11]. The high non-specific binding observed in PCT might correspond to the presence of a tubular carrier of low affinity for PK 11195, but this hypothesis calls for additional investigations. In addition, the distribution of specific [^{3}H]PK 11195 binding sites along the nephron is in good agreement with autoradiographic studies which showed radiolabelled ligand mainly in the glomeruli and in the outer medulla of rat kidney (Benavides, personal communication).

Previous studies, using membranes prepared from whole rat kidney, showed that the number of benzodiazepine receptors [3–5] was several-

Table 1

Apparent dissociation constants for binding of PK 11195 and benzodiazepines to medullary thick ascending limb (MAL) and medullary collecting tubule (MCT)

	MAL	MCT
PK 11195	2.2 nM	5.0 nM
Ro5-4864	4.9 nM	2.2 nM
Clonazepam	>30 μM	>30 μM

The apparent dissociation constants for the unlabelled compounds were calculated from I_{50} values; i.e., concentrations of drug leading to half-maximal inhibition of [^{3}H]PK 11195 specific binding

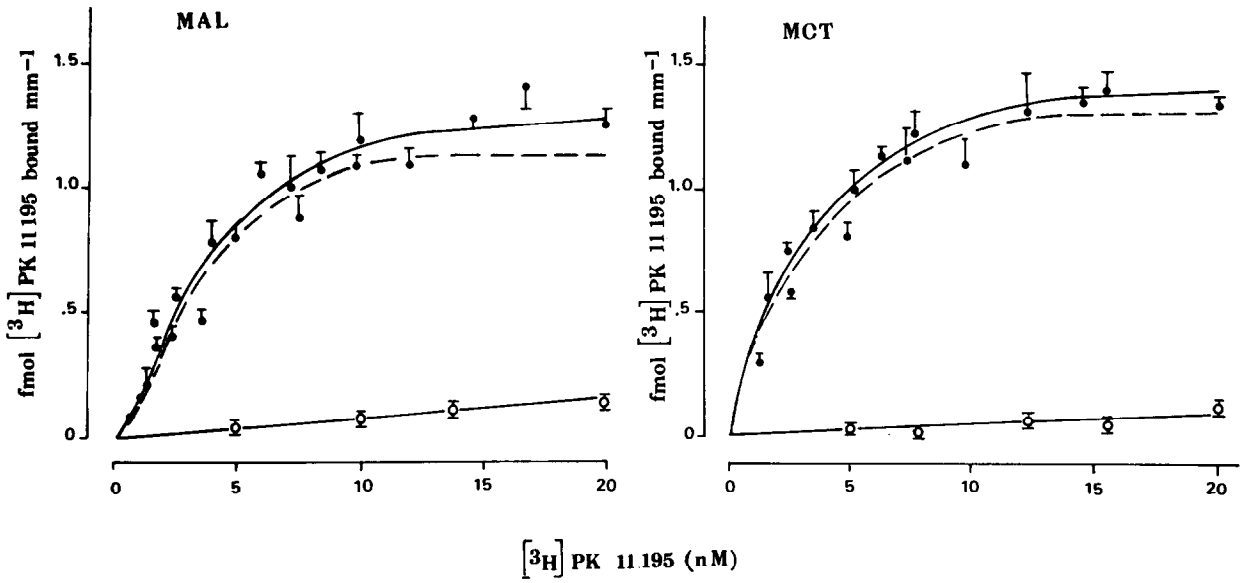

Fig.3. Dose dependency of total and non-specific binding of [^{3}H]PK 11195. Segments of medullary thick ascending limb (MAL, left) or medullary collecting tubule (MCT, right) were incubated with increasing amounts of [^{3}H]PK 11195 in the absence (total binding, ●—●) or presence (non-specific binding, ○—○) of 5 μM unlabelled PK 11195. Values are means ±SE of 3–5 determinations. Transformation of the specific component of the dose-dependent binding curves (– – –) in the Hill coordinates gives straight lines whose equations of linear regression are the following: MAL: $y = 1.71x + 14.55$ ($r = 0.962$, $P < 0.01$); MCT: $y = 1.56x + 13.46$ ($r = 0.921$, $P < 0.01$).

fold higher than that of the receptors to various hormones [12–14]. Here too, the maximal [^{3}H]PK 11195 specific binding capacity of the collecting tubule is about 100- and 200-fold higher than the respective numbers of aldosterone and insulin receptors [8,15]. Such a large concentration of peripheral benzodiazepine binding sites restricted to defined segments of the nephron raises

Table 2

[^{3}H]PK 11195 binding along the rat nephron

	Total binding	Non-specific binding	Specific binding
		(fmol bound per glomerulus)	
Glomerulus	0.38 ± 0.06 (7)	0.12 ± 0.07 (7)	0.26 ± 0.07*
		(fmol bound per nm)	
PCT	1.12 ± 0.55 (14)	0.63 ± 0.33 (17)	0.49 ± 0.44 NS
PR	0.35 ± 0.23 (7)	0.11 ± 0.09 (7)	0.24 ± 0.17 NS
MAL	1.26 ± 0.24 (18)	0.14 ± 0.06 (16)	1.12 ± 0.18*
CAL	1.86 ± 0.33 (8)	0.10 ± 0.09 (7)	1.76 ± 0.25*
CCT	1.25 ± 0.41 (7)	0.05 ± 0.06 (7)	1.20 ± 0.30*
MCT	1.24 ± 0.15 (15)	0.13 ± 0.08 (12)	1.11 ± 0.13*

Assays were performed at 20 nM [^{3}H]PK 11195. Results are means ± SD of n determinations (n, in parentheses). Quadratic ± SD values of specific binding were compared to zero using Student's t-test (NS, not significant; *, $P < 0.01$)

the obvious question about their function and biologic significance which are unknown. However, it has been shown that the number of renal benzodiazepine receptors was greater in rats of the Brattleboro strain with hereditary hypothalamic diabetes insipidus than that of normal rats of the same Long Evans hooded strain and that chronic treatment of Brattleboro with vasopressin returned this number of receptors to Long Evans control level [16]. In addition, the number of renal benzodiazepine receptors seems to be altered in hypertensive rats of the deoxycorticosterone/salt model [17,18]. It is worth noting that the distribution of benzodiazepine binding sites along the nephron covers the target cells of vasopressin on the one hand and those of aldosterone on the other. Vasopressin is known to induce contraction of glomerular mesangial cells through cyclic AMP-independent mechanisms [19] and to promote adenylate cyclase activation in the thick ascending limb of the Henle's loop and in the cortical and medullary portions of the collecting tubule [7], whereas aldosterone receptors are localized in the branched, cortical and medullary portions of the collecting tubule [15]. These observations might indicate a possible relationship between benzodiazepine receptors and vasopressin and mineralocorticoid actions in the kidney.

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