RESEARCH ARTICLE

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A COMPARATIVE ANALYSIS OF [^{3}H]-*PARA*-AMINOCLONIDINE BINDING TO RENAL TISSUES OF HYPERTENSIVE AND NORMOTENSIVE RATS

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ABSTRACT

A specific agonist for α_2-adrenoceptor, [^{3}H]-para-aminoclonidine ([^{3}H]-PAC), was used to characterize α_2-adrenoceptor binding in renal membranes from spontaneously hypertensive rat (SHR) and normotensive Wistar-Kyoto rat (WKY). The results from saturation and kinetic analyses revealed that two distinct binding sites, with relative high and low affinities, exist in the kidneys of both SHR and WKY. The K_d values for both binding sites were the same in SHR and WYK. However, the maximum receptor density (B_{max}) of both binding sites were higher in SHR than in WKY (32 ± 5.3 versus 22 ± 1.2 and 116 ± 12.5 versus 65 ± 2.0 fmol/mg protein for the high- and the low-affinity binding sites, respectively). GTP affected [^{3}H]-PAC binding to renal membranes from both SHR and WKY similarly and in a concentration-related manner. On the other hand, NaCl inhibited [^{3}H]-PAC binding to the low-affinity binding site to a lesser extent than to the high-affinity binding site in WKY, but both binding sites of SHR were similarly inhibited by NaCl. An increase in the receptor density of the renal membranes from SHR correlated well with their renal norepinephrine level and mean systolic blood pressure. Therefore, the data presented herein indicate that the alteration of [^{3}H]-PAC binding to α_2-adrenoceptor of the rat kidney is related to hypertension.

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INTRODUCTION

Over activation of renal nerve may play a role in the pathogenesis of hypertension, since it was demonstrated that renal denervation can delay onset of hypertension in spontaneously hypertensive rat of the Okamoto strain (SHR) and in deoxycorticosterone-acetate (DOCA)-salt rat.[1,2] Various adrenoceptor subtypes, including α_2-adrenoceptor are probably involved in the transmission of sympathetically mediated control of the renal function.[3-5] Several lines of evidence suggest that renal α_2-adrenoceptors modulate the release of renin from juxtaglomerular cells[6] as well as the reabsorption of sodium and water in the proximal convoluted tubules[7,8] and the collecting tubules.[9] Furthermore, the abnormality of α_2-adrenoceptors has been demonstrated in the hypertensive rats.[10,11]

Previous studies using the agonist [^{3}H]-clonidine, an antihypertensive drug,[4,12] and the antagonist [^{3}H]-yohimbine demonstrated only a single population of α_2-adrenoceptor in the rat kidney.[13,14] However, the pseudo-Hill coefficient of less than unity, obtained from drug competitive experiments, suggested two α_2-adrenoceptor subtypes. In contrast to the kidney, binding studies in the brain and other peripheral tissues have consistently reported the heterogeneity of the α_2-adrenoceptors.[15-17]

Due to the recent availability of [^{3}H]-*para*-aminoclonidine (PAC), which has a three-times-higher specific affinity for the α_2-adrenoceptor than [^{3}H]-clonidine,[18] it has been found that [^{3}H]-PAC can specifically bind, with high and low affinities, to the kidney of Sprague-Dawley rat,[19,20] and to the human platelets.[21] Therefore, [^{3}H]-PAC has been employed in the present study. Although an increase in renal α_2-adrenoceptor was previously reported in the SHR,[10] only a single binding was observed. It was the aim of this study to investigate the characteristics of specific [^{3}H]-PAC binding to α_2-adrenoceptors in the kidneys of spontaneously hypertensive rat (SHR) in comparison with the genetically related normotensive Wistar-Kyoto rat (WKY).

MATERIALS AND METHODS

Tissue preparation

Male spontaneously hypertensive rats (SHR) and their normotensive rats (WKY) (7 weeks old, Charles River Labs, USA) were used throughout the study. The animal systolic blood pressure was monitored by the tail-cuff method (Narco Biosystem, USA). After decapitation, the kidneys were rapidly removed and used for the crude membrane preparations according to the method of U'Prichard *et al.*[14] All the operations were carried out at 4°C, unless mentioned otherwise in each protocol. In brief, kidneys were homogenized with a Polytron homogenizer (Brinkman, Canada) in 20 volumes of ice-cold 50 mM tris(hydroxymethyl)aminomethane

(Tris)-HCl buffer (pH 7.6). The homogenate was centrifuged at 50,000 × g for 10 min and the resulting pellet was resuspended in the same buffer and recentrifuged. The final pellet was then resuspended in 50 volumes of 50 mM Tris-HCl (containing 10 mM $MgCl_2$, pH 7.6). The suspension generally contained a protein concentration of about 1-1.5 mg/ml. Protein concentration was determined by the Bio-Rad method,[22] using bovine gamma-globulin as the standard.

Radioligand binding assay

In general, the binding experiments were performed in triplicates using [^{3}H]-PAC (specific activity 42.0 Ci/mmol, New England Nuclear, USA) as a lignad. 500 μl of the crude membrane suspension was incubated for 35 min with [^{3}H]-PAC at 25° C in a total volume of 1 ml in 50 mM Tris-HCl buffer (containing 10 mM $MgCl_2$, pH 7.6). Blanks were incubated in the same manner with the addition of an excess of phentolamine (10 μM, Ciba-Geigy, USA) to determine the nonspecific binding. Specific binding was defined as the difference between total and nonspecific binding which was generally greater than 60% of the total binding. The incubation was terminated by vacuum filtration through Whatman GF/B filters using a Brandel tissue harvester. Following this, the filters were rapidly washed with 15 ml of ice-cold Tris-HCl buffer (pH 7.6) and placed in scintillation vials with 10 ml of Budget Solve (Researsh Products International, USA). The radioactivity retained on the filters was counted on a Beckman liquid scintillation spectrophotometer at 42% efficiency.

For saturation analysis, 0.1-20 nM [^{3}H]-PAC was used under the same assay conditions as described above. K_s (equilibrium dissociation constant) and B_{max} (maximum number of binding) values were estimated from the Scatchard plot[23] by the Rosenthal method.[24]

Although the results obtained from the Scatchard plot demonstrate two [^{3}H]-PAC binding sites to the renal membranes studied, it was very difficult to evaluate the characteristics of each binding site separately. Using the two different incubating temperatures, these two sites could not be differentiated in the rat kidneys, in contrast to that observed in the calf brain.[25] In order to partially observe the binding nature of each binding site, the experiment was performed at two different [^{3}H]-PAC concentrations (0.5 and 5 nM). These concentrations were below the K_{ds} of high- and low-affinity binbing sites respectively. At 0.5 nM, the binding reflects the majority of high-affinity site binding, whereas the results obtained from the assay at 5 nM represent the binding of both high- and low-affinity binding sites. Since the ratio of low:high affinity binding for the receptor density is about 3.6, the binding at 5 nM concentration of [^{3}H]-PAC should reflect the binding characteristics of the low-affinity binding site.

For kinetic analysis, association experiments were conducted by determining the time course of [^{3}H]-PAC binding. Dissociation experiments were performed by adding α-methylnorepinephrine as a displacer at different intervals of time after equilibrium binding was reached (usually 35 min at 25°C).

To study the effect of GTP and NaCl, the specific binding of [^{3}H]-PAC was investigated in the presence of GPT (1 μM - 1 mM) or NaCl (100-300 mM) under the conditions earlier described. The results were then expressed as percentages of specific binding in the absence of these modulators.

Norepinephrine determination

Renal norepinephrine concentrations were measured in the whole kidney by the alumina extraction method[26] and quantified by high pressure liquid chromatography with electrochemical detection.[27] Briefly, the renal tissue was rapidly homogenized in an appropiate volume of 0.1 N perchloric acid (2-5 ml). After centrifugation at 25,000 × g for 10 min, an aliquot of supernatant (200-400 μl) was added to 75-100 mg of activated alumina (Bioanalytical System, USA) in 1 ml of 0.1 M phosphate buffer (pH 7) with normethyldopamine (4 ng) as the internal standard. The pH was then adjusted to 8.6 by adding 3 M Tris buffer containing 2% EDTA. The samples were continuously shaken for 5 min, then the supernatant was aspirated and the alumina carefully washed twice with distilled water. The catecholamines were then eluted with 200-400 μl of 0.05 N perchloric acid by vigorous shaking and centrifugation through a 0.2 μm nylon filter. An aliquot of each sample was injected onto a Biophase-ODSC$_{18}$ reversed phase, 5 μm column (Bioanalytical System, USA) and eluted with mobile phase (14 mM citric acid, 6 mM sodium phosphate, 5% acetonitrile, 0.34 mM sodium octyl sulfate, pH 4.1). The values were corrected for recovery of the internal standard and expressed as ng/g wet weight kidney.

Chemicals

All chemicals were of analytical grade and obtained from Sigma or original sources as specified in each protocol.

Statistics

Results were expressed as mean ± SE. The data were statistically assessed by one-way analysis of variance and the significant difference for two point comparisons was analysed by unpaired Student's t-test.[28]

RESULTS

Saturation analysis

Specific binding of SHR renal membranes at increasing concentrations of [^{3}H]-PAC (0.1-20 nM) demonstrated a hyperbolic curve (Fig. 1). The Scatchard plot of these data is curvilinear which suggests two [^{3}H]-PAC binding sites to SHR kidney. The K_d and B_{max} values were 0.59 ± 0.05 nM and 32 ± 5.3 fmol/mg protein for the high-affinity binding site and 4.5 ± 0.08 nM and 116 ± 12.5 fmol/mg protein for the low-affinity binding site (n=3). The study from WKY renal membranes also revealed two [^{3}H]-PAC binding sites with K_d/B_{max} values of 0.5 ± 0.2 nM /22 ± 1.2 fmol/mg protein and 3.8 ± 0.05 nM/ 65 ± 2.0 fmol/mg protein for the high- and low-affinity binding sites, respectively (n=4).

Kinetic analysis

To confirm the existence of these two renal α_2-adrenoceptor binding sites, a kinetic analysis of [^{3}H]-PAC binding was performed. The association experiments were carried out at 25°C by incubating the SHR renal membrane for various lengths of time (5 sec - 45 min) with 0.5 nM and 5 nM [^{3}H]-PAC concentrations. The specific binding of [^{3}H]-PAC reached a plateau in 30 min (Fig. 2A). A semi-log plot of the association curve revealed a linear relationship with a slope of 0.09 min^{-1}.

The results of dissociation studies showed a biphasic manner of specific [^{3}H]-PAC binding to SHR renal membranes with rate constants of 0.25 min^{-1} and 0.023 min^{-1} for the fast and slow dissociations, respectively (Fig. 2B). The estimated K_d values from association and dissociation experiments[25] of these two components were 0.67 nM and 6.8 nM, corresponding to the high- and low-affinity binding sites from the Scatchard plot.

Effect of GTP and NaCl

α_2-adrenoceptors have been reported to inhibit the activity of adenylate cyclase and probably involve the GTP-binding protein as observed for the β-adrenoceptors, but in an opposive direction.[29,30] Published data on binding study have shown that GTP inhibits the binding of α_2-adrenoceptor agonist but stimulates the binding of α_2-adrenoceptor antagonist.[13,16] In the present study, GTP affects the [^{3}H]-PAC binding in a concentration dependent fashion (Fig. 3). At low concentrations (1 and 10 μM), GTP stimulated specific [^{3}H]-PAC binding to renal membranes from both SHR and WKY. However, at higher concentrations (100-1000 μM), GTP decreased the specific [^{3}H]-PAC binding in renal membranes from both SHR and WKY. The results from SHR studies seemed similar when assayed at either 0.5 nM or 5 nM [^{3}H]-PAC concentrations. Although GTP appeared to interfere with the [^{3}H]-PAC binding at 0.5 nM more than at 5 nM concentration, it was not statistically significant.

High sodium chloride diet was reported to participate in the pathogenesis of hypertension.[11] Moreover, the data from binding study demonstrated an inhibitory effect of NaCl on agonist binding.[31] In contrast to GTP, NaCl inhibited specific [^{3}H]-PAC binding to renal membranes from both SHR and WKY at every concentration assayed (100-300 mM) (Fig. 4). However, the inhibitory effect of NaCl is less in WKY than in SHR when assayed at the 5 nM [^{3}H]-PAC concentration.

Relation of receptor density to hypertension

To investigate whether or not the status of two [^{3}H]-PAC bindings to α_2-adrenoceptors is related to hypertension, the renal norepinephrine level and the mean systolic blood pressure of SHR and WKY were monitored. As summarized in Table 1, the receptor densities of both high- and low-affinity [^{3}H]-PAC binding sites significantly increased in SHR compared to in WKY. The increase correlated with the renal norepinephrine level and mean systolic blood pressure.

DISCUSSION

Many recent studies have clearly demonstrated that the renal sympathetic nerves are important in the development and maintenance of hypertension in several animal models.[1,2] An increase of adrenoceptor density in the kidney may contribute to the pathogenesis of hypertension by inducing an inappropiate renal sodium retention.[3,5,6] Past studies also reported the increase of α_2-adrenoceptor density in the kidneys of Dahl-salt sensitive rats SHR,[10,11] but these studies revealed only a single α_2-adrenoceptor of the kidneys. The present study confirms an increase in α_2-adrenoceptor density in the SHR kidney as previously reported, and, moreover, demonstrates that both high- and low-affinity [^{3}H]-PAC binding sites of α_2-adrenoceptors are present in the SHR and WKY kidneys. Only the receptor density, and not the receptor affinity, of these α_2-adrenoceptors were elevated in SHR as compared to WKY. The increase in both renal norepinephrine and α_2-adrenoceptor density in SHR in this study supports the notion that over activity of the sympathetic nerve plays a role in hypertension. GTP showed similar inhibition on [^{3}H]-PAC binding to both high- and low-affinity binding sites in SHR and WKY, but the low-affinity binding site of WKY was significantly more resistant to NaCl inhibition than their high-affinity binding site. Similar inhibition was observed for high- and low-affinity binding sites in SHR.

The existence of high- and low-affinity binding sites for the binding of [^{3}H]-PAC to renal α_2-adrenoceptors has also been reported in the kidney of Sprague-Dawley rat.[19,20] The difference in results obtained from this study and the previous report possibly reflects two things. First, because of the higher affinity of [^{3}H]-PAC used in this study, it is a more appropiate ligand than [^{3}H]-clonidine and [^{3}H]-

yohimbine, both of which may fail to detect the low-affinity binding site. Second, whole kidneys were used for renal membrane preparation in this study, whereas only the cortical membranes were used in the previous studies.[13,14] The localization α_2-adrenoceptors has been shown to be at both the cortex and medulla of the rat kidney, by using both agonist [^{3}H]-PAC ad antagonist [^{3}H]-idazoxan.[34] Moreover, McPherson and Summers previously reported [^{3}H]-clonidine binding to both cortex and medulla in the rat kidney but not in the guinea pig kidney.[12]

By using two different [^{3}H]-PAC concentrations as earlier described in the method, it was found that GTP has similar effects on the [^{3}H]-PAC binding to the kidneys of both SHR and WKY, in contrast to the selective effect on the high-affinity binding of the brain.[16] This could account for the tissue difference of α_2-adrenoceptors.

NaCl inhibited the binding of the low-affinity site less than the high-affinity site in WKY, but inhibited both the high- and low-affinity binding sites in SHR. In other words, the low-affinity binding site in SHR seems to be more sensitive to salt inhibition than that in WKY. The increase in receptor density of the low-affinity binding site in SHR may have resulted from an up-regulation mechanism to compensate for the response to salt. This is not an advantage, but rather to potentiate the pathology of hypertension. However, the validity of this hypothesis will require further research.

Many functional roles have been proposed for the renal α_2-adrenoceptors. For instance, Young and Kuhar[32] suggested that α_2-adrenoceptors may be involved in the sodium reabsorption since they found that [^{3}H]-clonidine binds preferentially to the proximal tubule of the guinea pig kidney. In the dog, the α_2-adrenoceptors are confined to the glomeruli.[33] In the rat, we have previously demonstrated the greater binding of [^{3}H]-PAC to the medulla than to the cortex by both autoradiography and membrane binding assay.[34] Therefore, the distribution of renal α_2-adrenoceptors seems to be species specific and the receptors at different locations may act differently. Umemura *et al*[8] have demonstrated that activation of α_2-adrenoceptors can inhibit adenylate cyclase activity stimulated by parathyroid hormone in the proximal tubules and by arginine vasopressin in the cortical and medullary collecting tubules of the rat kidney. Furthermore, activation of rat renal α_2-adrenoceptors by epinephrine infusion in the presence of β- and α_2-adrenoceptor blockade resulted in inhibition of adenylate cyclase activity and sodium retention stimulated by furosemide.[35] In addition, α_2-adrenoceptor activation also reversed vasopressin-mediated changes in water and sodium excretion in the isolated perfused kidney of the rabbit.[36] Taken together, the renal α_2-adrenoceptors may regulate several adenylate cyclase activation phenomena including sodium reabsorption at the proximal tubules, water and electrolyte excretion at the collecting tubules. In

certain hypertensive animals such as the SHR and DOCA-salt rats, over activity of the renal nerve is likely to result in imbalance of renal function. Therefore, the abnormality of renal nerve activity, such as the increase in α_2-adrenoceptors and changes in their response to salt inhibition, as observed in this sutdy, could be an important factor in the pathogenesis of hypertension.

In summary, the above data demonstrate that two [^{3}H]-PAC binding affinities are present in the kidneys of SHR and WKY. Only the receptor density, not the receptor affinity, of the two binding sites was increased in SHR. These increases exhibited good correlations with the level of renal norepinephrine and mean systolic blood pressure. GTP could not differentiate the [^{3}H]-PAC binding of either high- or low-affinity binding sites, while NaCl inhibited low-affinity binding sites in the WKY to a lesser extent than in the SHR.

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บทคัดย่อ

จากการศึกษาเปรียบเทียบการจับของ [^{3}H]-PAC ซึ่งเป็นอนุพันธุ์ของยาลดความดันโลหิตสูง ชนิดที่มีความเฉพาะต่อรีเซพเตอร์ α_2-adrenoceptors ของเมมเบรนที่สกัดจากไตของหนูที่มีความดันโลหิตสูงและหนูปกติ พบว่า [^{3}H]-PAC จับกับเมมเบรนจากหนูทั้ง 2 กลุ่มด้วย 2 affinity binding sites ค่า B_{max} ของรีเซพเตอร์ในกลุ่มหนูที่มีความดันโลหิตสูงมีค่าสูงกว่าในหนูปกติ คือ 32 ± 5.3 ต่อ 22 ± 1.2 fmol/mg protein สำหรับ high-affinity binding site และ 116 ± 12.5 ต่อ 65 ± 2.0 fmol/mg protein สำหรับ low-affinity binding site โดยที่ค่า K_d ไม่แตกต่างกัน GTPในปริมาณต่ำมีผลกระตุ้นการจับของ [^{3}H]-PAC แต่ในปริมาณสูงกลับมีผลยับยั้ง คล้ายกันในทั้ง 2 กลุ่มของหนู สำหรับเกลือโซเดียมคลอไรด์มีผลยับยั้งการจับของ [^{3}H]-PAC โดยที่มีผลยับยั้งต่อ low-affinity binding site ของหนูที่มีความดันโลหิตสูงมากกว่าต่อหนูปกติ นอกจากนี้พบว่าหนูที่มีความดันโลหิตสูงมีจำนวนรีเซพเตอร์และระดับของ norepinephrine ของไตสูงกว่าหนูปกติ

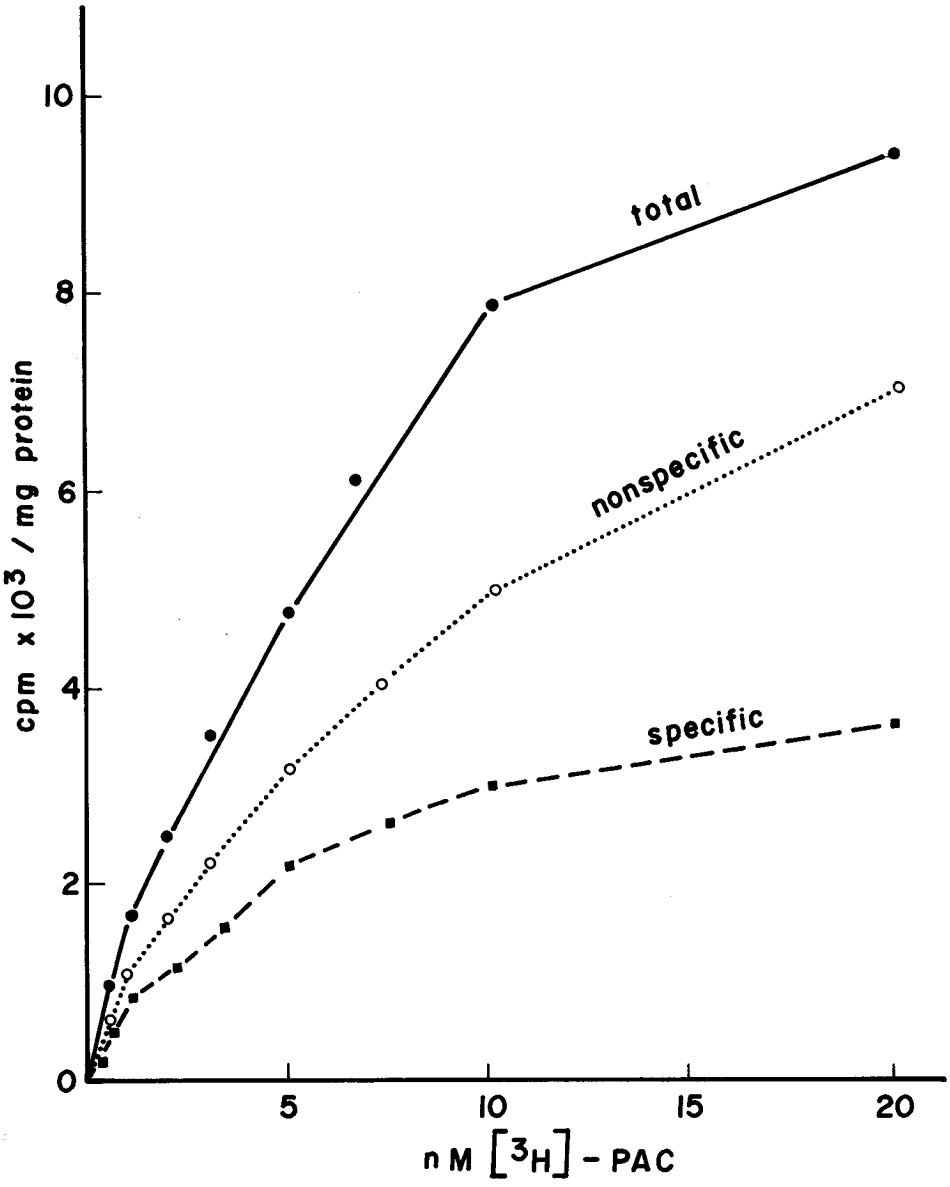

Fig. 1 [^{3}H]-PAC binding at increasing concentrations of radioligand. SHR renal membranes were incubated at 25°C for 35 min at various concentrations of [^{3}H]-PAC (0.1-20nM). Points represent the average of triplicate determinations.

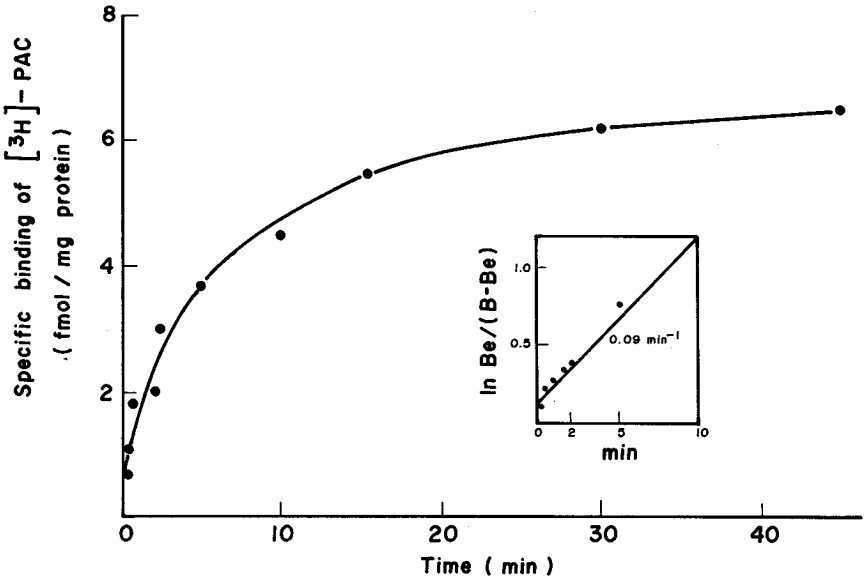

Fig.2A Association of [^{3}H]-PAC specific binding to renal membrane from SHR was determined at various times following an addition of 0.5 nM [^{3}H]-PAC. The specific binding at each point is the average from triplicate assays. The specific binding reached a plateau by 30 min. Inset : A semilog plot of pseudo-first order kinetics of association. B_e = specific binding at equilibrium, B = specific binding at the time point shown.

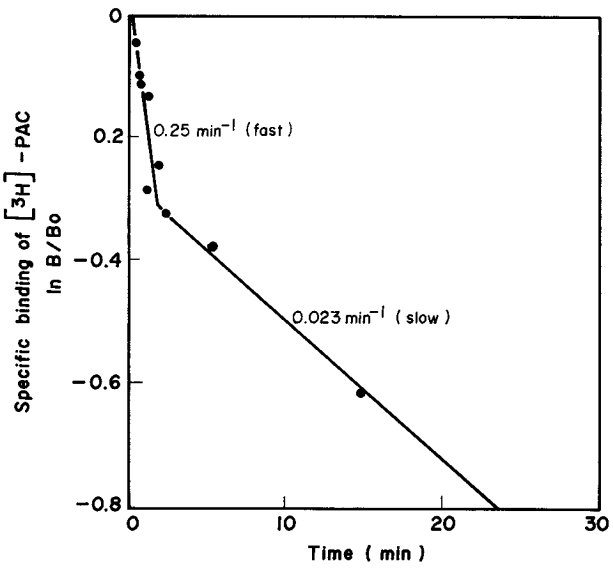

Fig.2B Dissociation of [^{3}H]-PAC specific binding at 25°C. After 35 min preincubation of 0.5nM [^{3}H]-PAC with renal membranes from SHR, 10 μM of α-methylnorepinephrine was added to the incubation mixtures and the reactions were terminated by rapid filtration at various indicated times. B_0 is specific binding at time zero before addition of displacer, B is specific binding at the time point indicated. Dissociation was biphasic displaying both a fast and a slow component.

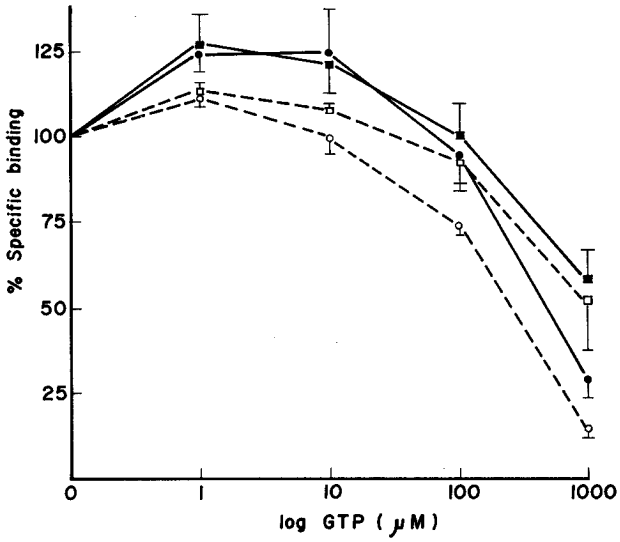

Fig.3 Effect of guanosine triphosphate on the specific binding of [^{3}H]-PAC to renal membranes. Specific binding was determined by incubating renal membranes with either 0.5nM (● SHR, ○ WKY) or 5nM (■ SHR, □ WKY) for 35 min in the presence of GTP (n=3).

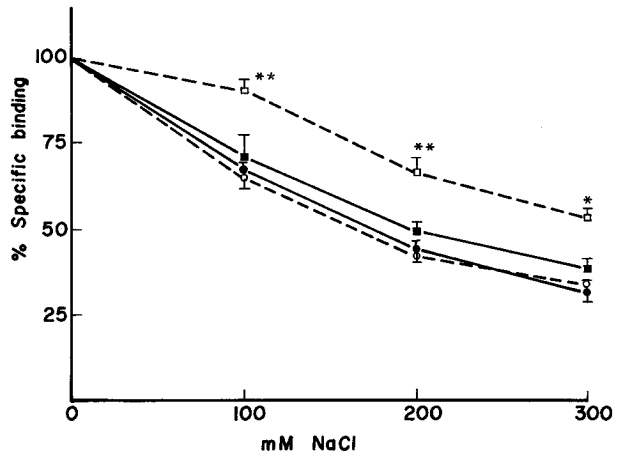

Fig. 4 Effect of sodium chloride on the specific binding of [^{3}H]-PAC to renal membranes. At 5 nM concentration, NaCl inhibits [^{3}H]-PAC binding in WKY (□) less than in SHR (■), whereas at 0.5nM concentration, the inhibitory effect of NaCl on [^{3}H]-PAC binding is the same in SHR (●) or WKY (□).(* = $p < 0.02$ and ** = $p < 0.05$, n = 3).

TABLE 1 Comparision of [^{3}H]-PAC binding, renal norepinephrine content and mean systolic blood pressure in hypertensive and normotensive rats at 7 weeks of age.

group	binding of [^{3}H]-PAC (fmol/mg protein) (n = 10)		renal norepinephrine level	mean systolic blood pressure (mmHg, n = 18)
	at 0.5nM	at 5 nM	(ng/g, n = 8)	
SHR	16.3 ± 1.5*	73.6 ± 3.2**	274 ± 10**	162 ± 3.4**
WKY	10.5 ± 0.9	52.2 ± 2.7	186 ± 6	135 ± 1.7

* significantly different from WKY at $p < 0.01$.

** significantly different from WKY at $p < 0.001$.