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[^{3}H]-CLONIDINE BINDING TO α-ADRENOCEPTORS IN MEMBRANES PREPARED FROM REGIONS OF GUINEA-PIG KIDNEY: ALTERATION BY MONOVALENT AND DIVALENT CATIONS

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1 [^{3}H]-clonidine binds reversibly to membranes prepared from regions of guinea-pig kidney.

2 Higher levels of binding were obtained in the membranes prepared from renal cortex (2.15 ± 0.27 pmol/g wet wt.) than renal medulla (0.53 ± 0.07 pmol/g wet wt.) or papilla (0.14 ± 0.06 pmol/g wet wt.; $n = 4$).

3 Scatchard analysis performed by addition of unlabelled clonidine (1 to 30 pmol) gave figures for the dissociation constant (K_d) for the binding of [^{3}H]-clonidine to renal cortical membranes of 9.0 ± 0.8 nM and B_{max} of 21.6 ± 1.7 pmol/g wet wt. ($n = 4$). Hill plots of these data gave gradients close to unity, indicating a lack of co-operative site interactions.

4 The monovalent cations, sodium and potassium, and the divalent cation, calcium, produced concentration-dependent decreases in [^{3}H]-clonidine binding to membranes prepared from renal cortex, the EC_{50}s being respectively 25 mM, 37 mM and 23 mM.

5 At low concentrations the divalent cations, magnesium (1 mM) and manganese (0.1 mM), produced enhancement of [^{3}H]-clonidine binding. At higher concentrations (>10 mM) both divalent cations inhibited binding.

6 Scatchard analysis of [^{3}H]-clonidine binding performed in the presence of sodium (100 mM), magnesium (1 mM) or manganese (0.1 mM) revealed that the alterations in binding are primarily due to changes in apparent affinity rather than a change in the number of binding sites. Sodium (100 mM) produced a change in the K_d from 7.0 ± 0.4 nM ($n = 8$) to 42.3 ± 27.5 nM ($n = 3$), whereas magnesium (1 mM) decreased the K_d to 6.0 ± 0.9 nM and manganese (0.1 mM) to 4.0 ± 1.0 nM ($n = 3$).

7 The results indicate that [^{3}H]-clonidine labels a binding site that has properties resembling an α_2-adrenoceptor, located in the renal cortex. The changes produced by the addition of monovalent and divalent cations are entirely due to changes in the apparent affinity of [^{3}H]-clonidine binding.

Introduction

The use of [^{3}H]-clonidine to identify α-adrenoceptors in brain (U'Prichard, Greenberg & Snyder, 1977; Jarrott, Louis & Summers, 1979a; U'Prichard, Bechtel, Rouot & Snyder, 1979; Glossmann & Presek, 1979; Jarrott, Summers, Culvenor & Louis, 1980) and in peripheral tissues such as guinea-pig kidney (Jarrott & Summers, 1978; Jarrott, Louis & Summers, 1979b), rat kidney, submaxillary gland, spleen and rabbit duodenum, (U'Prichard & Snyder, 1979) is now well established. Pharmacological characterization of the clonidine binding by displacement studies indicates that it is to a site resembling an α_2-adrenoceptor in both rat cerebral cortex and guinea-pig kidney (U'Prichard *et al.*, 1977; 1979; Jarrott *et al.*, 1979b) and judged from denervation studies with 6-hydroxydopamine, the binding site in both tissues is located postsynaptically (U'Prichard & Snyder, 1979, Jarrott & Summers, unpublished observations).

Examination of the regional localization of the clonidine binding site in rat brain has revealed a widespread distribution of α_2-adrenoceptors with the highest concentration in membranes prepared from cerebral cortex and hypothalamus and the lowest in those prepared from cerebellum (U'Prichard *et al.*, 1979). Kinetic analysis of binding revealed that both rat brain and guinea-pig kidney have a similar density of binding sites and that membranes from both tissues bind [^{3}H]-clonidine by a high affinity process (U'Prichard *et al.*, 1977; Jarrott *et al.*, 1979b). In addition, in both sites binding displays neither positive nor negative cooperativity indicating a simple monomolecular reaction.

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In a number of different tissues, monovalent and divalent cations are known to modulate binding of agonists to α-adrenoceptors. High concentrations of sodium inhibit, whereas low concentrations of magnesium and manganese enhance binding in membranes from rat and calf cerebral cortex and lysates of rabbit platelets (Greenberg, U'Prichard, Sheehan & Snyder, 1978; Tsai & Lefkowitz, 1978; Glossmann & Presek 1979; Hornung, Presek & Glossmann, 1979). In calf cerebral cortex (Greenberg *et al.*, 1978) it has been suggested that the sodium effect is due to a reduction in the number of receptor sites, whereas in platelet lysates it is due to changes in affinity (Tsai & Lefkowitz, 1978).

In the study to be described, the regional localization of the binding sites in guinea-pig kidney membranes has been examined together with the alteration of their properties by cations. A preliminary account of some of the results has been published (Jarrott & Summers, 1978).

Methods

Dissection of kidney regions

Male guinea-pigs (500 to 800 g) were anaesthetized with halothane and rapidly killed by exsanguination. The kidneys were excised and placed on ice. The outer connective tissue capsule was removed and the kidney sliced along its longitudinal axis with a razor blade. Three regions could readily be discerned: the papilla nearest the hilum, fanning out to the darker medulla which in turn showed a distinct boundary with the pale brown cortex. The areas were dissected with a scalpel blade and strabismus scissors.

Radioligand binding assay

Membranes were prepared by homogenization in 20 vols. 50 mM Tris-HCl buffer (pH 7.6 at 4°C) and centrifugation at 49,000 *g* as described by U'Prichard *et al.* (1977).

The membrane suspension was incubated in Tris buffer (50 mM, pH 7.6 at 25°C) with [^{3}H]-clonidine (5.29 Ci/mmol in earlier experiments) to give a concentration of 2.5 mM and with [^{3}H]-clonidine (26.7 Ci/mmol in later experiments) to give a concentration of 1 nM. The total [^{3}H]-clonidine bound was always less than 2.5% of the total label added. Non-specific binding was estimated in samples containing 10 μM phentolamine. Similar values for non-specific binding were also obtained with 1 μM clonidine or 10 μM (−)-noradrenaline. After incubation, membranes were harvested, washed and the label eluted as previously described (Jarrott *et al.*, 1979b). Filtration was facilitated by the use of a filtration manifold modified from that described by Anderson, Berg & Rossi, (1975). The base of the manifold consists of the bottom half of a 250 mm diameter polypropylene dessicator. A piece of 3/8 or 1/2 inch thick perspex is cut to fit inside the flange of the dessicator base so that it seals against a rubber O-ring. Holes are drilled and reamed in the perspex to accept standard luer adaptors. Millipore Swinnex Filter holders (Millipore Cat No. SX00 025 00) fit directly into these luer fittings. The pattern adopted for the drilling of the holes allows the Swinnex Filters to be hexagonally close packed so that 36 holders can be mounted on the plate leaving one hole to apply vacuum and another to fit a 3-way tap to release vaccum at the end of filtration. The Whatman GF/B filters were soaked in distilled water and mounted in the holders without gaskets. A syringe barrel from a disposable 5 ml luer lock syringe is then mounted on top of the Swinnex holder to act as a funnel for the wash solution. This system offers the advantage of being easy to make from cheap, readily available materials and has been in use in this department for the last 3 years.

Calculation of results

Scatchard and Hill analysis and the calculation of K_i values for displacers of [^{3}H]-clonidine binding were performed as previously described (Jarrott *et al.*, 1979*b*).

Drugs

Drugs were prepared as previously described (Jarrott *et al.*, 1977b). The following drugs were used: [^{3}H]-clonidine (5.29 Ci/mmol prepared by Dr M. Delaney and Dr A. Lieberman, Roche); [^{3}H]-clonidine (26.7 Ci/mmol; a kind gift from Boehringer Ingelheim). The author gratefully acknowledges the gift of clonidine hydrochloride (Boehringer). (−)-Noradrenaline bitartrate was obtained from Sigma. The chloride salts of sodium, potassium, calcium, magnesium and manganese were all Analar grade chemicals.

Results

Regional localisation of [^{3}H]-clonidine binding

Kidneys were removed from male guinea-pigs and the renal cortex, medulla and papilla separated by dissection along the lines shown in Figure 1. The dissection was carried out at 4°C. Membranes were prepared from each area as previously described (Jarrott *et al.*, 1979b) and incubated with [^{3}H]-clonidine (5.29 Ci/mmol; approx. 2.5 nM) for 30 min at 25°C in a shaking water bath. After filtration, washing and elution of bound [^{3}H]-clonidine it was evident that much

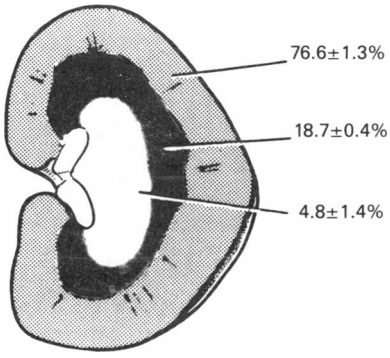

Figure 1 Diagram drawn from a longitudinal section of guinea-pig kidney showing the papilla (inner white area), medulla (dark shading), and cortex (outer stippled area). The numbers refer to the percentage of total binding obtained in membranes prepared from the three areas; ($n = 4$). Incubation conditions: 1 ml of membrane suspension containing 20 mg wet wt. tissue, 1 ml 50 mM Tris buffer pH 7.6 at 25°C, incubation for 30 min at 25°C.

higher specific binding levels occurred to membranes prepared from renal cortex (2.15 ± 0.27 pmol/g wet wt.) than to renal medulla (0.53 ± 0.07 pmol/g wet wt.) or papilla (0.14 ± 0.06 pmol/g wet wt, $n = 4$) as shown in Figure 1. These values were obtained from incubations carried out simultaneously using the same volume of membranes (1 ml of membrane suspension containing 20 mg wet wt. tissue).

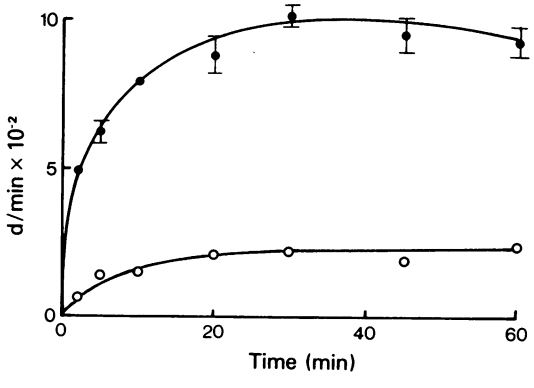

Figure 2 Time course of specific [^{3}H]-clonidine binding to membranes prepared from guinea-pig kidney cortex (●; $n = 6$), and medulla (○; $n = 2$). [^{3}H]-clonidine concentration ~2.5 nM, incubation temperature 25°C. Specific binding is defined as the difference between total [^{3}H]-clonidine binding and that in the presence of 10 μM phentolamine. Vertical bars indicate s.e. mean.

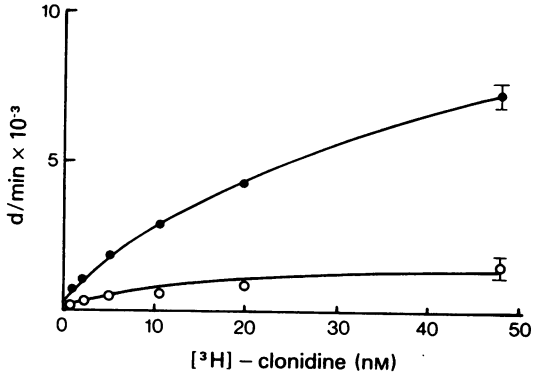

Figure 3 Saturation of specific [^{3}H]-clonidine binding to membranes prepared from guinea-pig kidney cortex (●; $n = 6$), and medulla (○; $n = 4$). [^{3}H]-clonidine concentration ~2.5 nM, incubation temperature 25°C. Vertical bars indicate s.e. mean.

Time course of specific [^{3}H]-clonidine binding to membranes from renal medulla and cortex

Membranes prepared from the two areas of the kidney which displayed a significant degree of binding (i.e. medulla and cortex) were studied in greater detail. The time course of [^{3}H]-clonidine binding in membranes from the two areas revealed that association to the binding site was rapid and that equilibrium was reached within 20 min of incubation (Figure 2). The maximum level reached in membranes prepared from renal cortex was over four times that in membranes from renal medulla.

Saturation of specific [^{3}H]-clonidine binding

Specific [^{3}H]-clonidine binding to membranes prepared from guinea-pig cortex and medulla was saturable as shown by the binding isotherms in Figure 3, which show evidence of reaching a plateau at concentrations of [^{3}H]-clonidine greater than 50 nM when incubated from 30 min at 25°C. There was no evidence of heterogeneity of binding.

Saturation analysis of [^{3}H]-clonidine binding to membranes from cortex and medulla

Scatchard analysis of [^{3}H]-clonidine binding to membranes from the two areas was carried out as previously described (Jarrott *et al.*, 1979b). After incubation under equilibrium conditions it could be seen that binding of [^{3}H]-clonidine is of high affinity to a single population of binding sites in both areas. The results of typical experiments are shown in Figure 4 together with (shown as a broken line for comparison), data previously obtained with membranes pre-

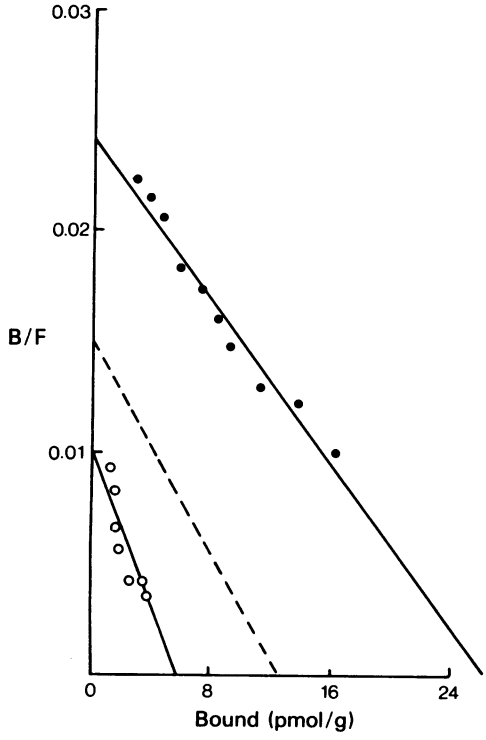

Figure 4 Scatchard plots of data obtained from typical experiments by addition of increasing amounts of clonidine (1 to 30 pmol) to membrane preparations from guinea-pig renal cortex (●), medulla (○) and whole kidney (broken line). [^{3}H]-clonidine concentration ~2.5 nM.

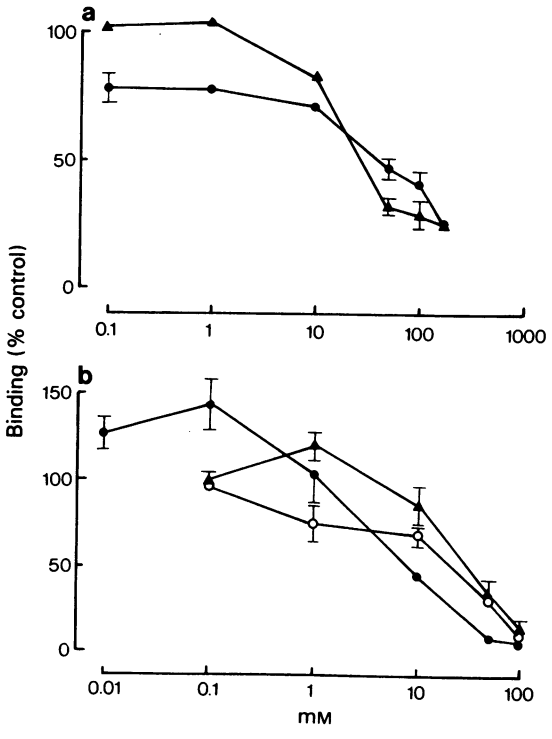

Figure 5 The effect of monovalent and divalent cations on [^{3}H]-clonidine binding in membranes prepared from guinea-pig renal cortex. In (a) effect of addition of sodium (▲) and potassium (●) ions (n = 3); (b) effect of addition of manganese (●), magnesium (▲) and calcium ions (○) (n = 4). Incubation conditions: [^{3}H]-clonidine concentration ~1 nM, incubation temperature 25°C for 30 min. Error bars indicate s.e. mean.

pared from whole kidney (Jarrott *et al.*, 1979b). This type of analysis was carried out in all experiments and the pooled data gave values for K_d of 9.0 ± 0.8 nM and B_{max} of 21.6 ± 1.7 pmol/g wet wt. (n = 4) for renal cortical membranes and a K_d of 4.4 ± 0.9 nM and B_{max} of 3.9 ± 1.0 pmol/g. wet wt. (n = 3) for renal medulla membranes. It can be seen from a comparison of these figures with those obtained in whole kidney that the separation into regions provides evidence that most of the renal α-adrenoceptors are located in the renal cortex.

Hill plots of the data obtained in these experiments were linear ($P < 0.001$ in all cases) with a mean Hill co-efficient of 1.02 ± 0.02 (n = 4) indicating that there was no cooperativity involved in the binding as has been previously shown for whole kidney (Jarrott *et al.*, 1979b).

Effects of sodium and potassium ions on [^{3}H]-clonidine binding

Monovalent cations are known to modulate agonist binding to membranes prepared from cerebral cortex and platelets (Greenberg *et al.*, 1978; Tsai & Lefkowitz, 1978; Glossmann & Presek, 1979; Hornung *et al.*, 1979). In membranes prepared from guinea-pig renal cortex, both sodium and potassium produced concentration-dependent decreases in [^{3}H]-clonidine binding which were marked at concentrations of sodium and potassium ions greater than 10 mM (Figure 5a). The concentrations of sodium and potassium ions which produced a 50% reduction in binding were respectively 25 mM and 37 mM.

Effects of magnesium, manganese and calcium ions on [^{3}H]-clonidine binding

Addition of calcium ions to the incubation medium produced dose-dependent inhibition of [^{3}H]-clonidine binding which was marked at concentrations greater than 10 mM (Figure 5b). The effect of the other two divalent cations magnesium and manganese was more complex. Manganese (0.1 mM) or magnesium (1

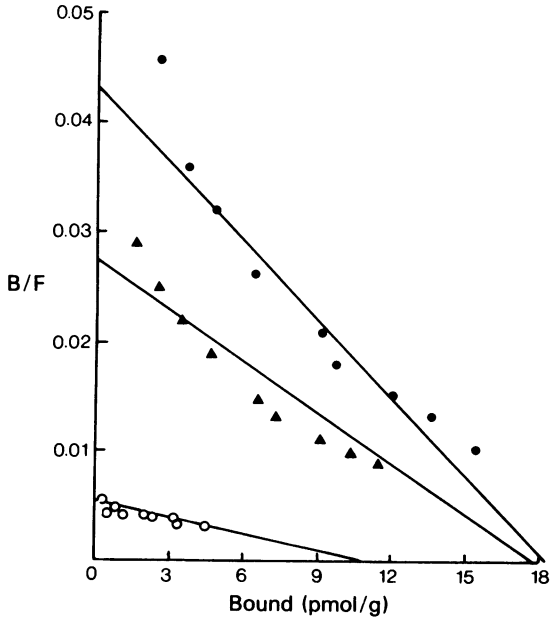

Figure 6 Scatchard plots of data obtained from typical experiments by addition of increasing amounts of clonidine (1 to 30 pmol) to membrane preparations of guinea-pig renal cortex; (●) in the presence of 0.1 mM manganese, (▲) in the presence of 1 mM magnesium and (○) in the presence of 100 mM sodium ions. K_d for Mn^{2+} 4.0 nM, for Mg^{2+} 6.4 nM and for Na^+ 21.9 nM.

mM) produced a significant enhancement of binding which was followed at higher concentrations by a dose-dependent inhibition.

The effects of sodium (100 mM), magnesium (1 mM) and manganese (0.1 mM) were studied in more detail. Scatchard plots of saturation experiments carried out in the presence of these ions are shown in Figure 6. It can be seen that the alterations in binding that all three cations produce are due to changes in apparent affinity rather than any change in the number of binding sites (Table 1). Sodium produced a decrease in affinity, the K_d changed from 7.0 ± 0.4 nM to 42.3 ± 27.5 nM ($P < 0.025$, d.f. = 9) whereas magnesium changed the K_d to 6.0 ± 0.9 nM (ns) and manganese to 4.0 ± 1.0 nM ($P < 0.005$, d.f. 9). The slight curvilinearity observed in some of the Scatchard plots (Figure 6) was not due to negative cooperativity since the Hill co-efficients in all experiments remained not significantly different from unity.

Discussion

This paper describes the binding of [^{3}H]-clonidine to membranes prepared from regions of guinea-pig kidney. Since the number of binding sites obtained in membranes prepared from renal cortex was almost twice that in membranes from whole kidney, it is evident that most renal α-adrenoceptor-like binding sites are localized in the renal cortex. There were only 1/5th the number of the binding sites in membranes prepared from renal medulla and it is possible that this could represent contamination from the cortex. However the K_d obtained in the medullary membranes was about half of that in the cortical membrane so that it could represent binding to a distinct site. It would of course be desirable to determine the precise binding site within the cortex and medulla and experiments designed to examine this point are in progress.

It has previously been suggested (Jarrott *et al.*, 1979b) on the basis of the relative potency of drugs acting on [^{3}H]-clonidine binding and those which affect renin release that the α-adrenoceptor in kidney membranes may resemble that which inhibits renin release (Pettinger, Keeton, Campbell & Harper, 1976). Since renin release is from the juxtaglomerular apparatus located in the renal cortex the present findings are not in disagreement with this view. However, it must be borne in mind that there are other candidates for the binding site including the α-adrenoceptors

Table 1 Effects of sodium, magnesium and manganese ions on kinetic parameters of [^{3}H]-clonidine binding to membranes prepared from guinea-pig renal cortex

Treatment	K_d(nM)	B_{max} pmol/g wet wt.	*nH*	n
Control	7.0 ± 0.4	17.2 ± 0.7	1.002 ± 0.004	8
Na^+ 100 mM	42.3 ± 27.5*	19.1 ± 9.2	0.99 ± 0.01	3
Mg^{2+} 1 mM	6.0 ± 0.9	18.3 ± 2.4	1.005 ± 0.01	3
Mn^{2+} 0.1 mM	4.0 ± 1.0**	19.2 ± 3.1	1.031 ± 0.006	3

*$P < 0.025$ cf. control; **$P < 0.005$ cf. control.

involved in gluconeogenesis in renal tubules (Guder & Rupprecht, 1975; MacDonald & Saggerson, 1977) and α-adrenoceptors in blood vessels.

In the previous study with membranes from the whole kidney, displacement studies using a wide variety of drugs indicated that the [^{3}H]-clonidine binding was exclusively to an α-adrenoceptor since drugs acting on β_1- and β_2-adrenoceptors, histamine H_1- and H_2-receptors, nicotinic acetylcholine receptors, muscarinic acetylcholine receptors, opiate receptors and prostaglandin receptors had no effect. Both α-adrenoceptor agonists and antagonists displaced binding, with agonists being generally more potent (Jarrott *et al.*, 1979b).

Binding of agonists to α-adrenoceptors in other tissues is known to be affected by the presence of certain cations. Sodium ions inhibit agonist binding in rabbit platelet lysates and in rat and bovine cerebral cortex membranes (Greenberg *et al.*, 1978; Tsai & Lefkowitz, 1978; Glossmann & Presek, 1979; Hornung *et al.*, 1979). In rabbit platelet lysates and in the present study using renal cortical membranes, the effect is due entirely to a decrease in the apparent affinity of binding whereas in bovine cerebral cortex membranes the decreased binding appears to be due to a decrease in the number of agonist sites (Greenberg *et al.*, 1978). Subsequently, however, it has been suggested that in bovine cerebral cortex membranes sodium may reduce the affinity of the majority of sites to such a low level that it is not easily discernible but leave a small number of high affinity sites unaffected so giving the impression that the number of sites has been reduced (U'Prichard & Snyder, 1978). In rat cerebral cortex, sodium produces the decrease in binding by inducing negative cooperativity (Glossmann & Presek, 1979) but no change in cooperativity was noted in guinea-pig renal cortex membranes. The effect of potassium ions on [^{3}H]-clonidine binding in renal membranes is different from that reported in other tissues. In cerebral cortex potassium ions have relatively little inhibitory effect on binding (Greenberg *et al.*, 1978) whereas in rabbit platelets at equal econcentrations (100 mM) potassium is only 19% as effective as sodium (Tsai & Lefkowitz, 1978). In renal cortical membranes, potassium and sodium at a concentration of 100 mM produced a similar 60 to 70% decrease in binding.

The divalent cation calcium at low concentrations (~1 mM) has little effect on binding in any of the systems that have been studied (Tsai & Lefkowitz, 1978; U'Prichard & Snyder, 1978; Hornung *et al.*, 1979). In the present study calcium inhibited binding only at concentrations greater than 10 mM. Magnesium and manganese at low concentrations are known to enhance binding of agonists to membranes from rat and calf cerebral cortex and rabbit platelets. In this latter preparation (Tsai & Lefkowitz, 1978) and in renal cortical membranes the effect of these ions is due to an increase in apparent affinity of binding. At high concentrations both divalent cations produced a pronounced inhibition of binding.

It is interesting to speculate on the significance of the effect of ions on binding. Certainly the ions vary markedly in their effects and in the concentration required to produce the effect. Near physiological concentrations of sodium (100 mM) produce a profound decrease in affinity of binding which could indicate that the receptor is linked to a sodium transport system. The enhancing effect of magnesium on binding is also seen at near physiological levels perhaps indicating that agonist binding to adrenoceptors is normally enhanced by the presence of this divalent cation.

In conclusion, these observations show that [^{3}H]-clonidine binding is to a site located primarily in the renal cortex. Binding is affected by the presence of monovalent and divalent cations and the alterations in binding are due entirely to changes in apparent affinity rather than to changes in the number of receptor sites.

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