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INACTIVATION OF [^{125}I]-ANGIOTENSIN II BINDING SITES IN RAT RENAL CORTEX EPITHELIAL MEMBRANES BY DITHIOTHREITOL

HELEN M. COX,* KENNETH A. MUNDAY and JUDITH A. POAT
Department of Physiology and Pharmacology, University of Southampton, Southampton SO9 3TU, U.K.

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Abstract—Preincubation of renal epithelial membranes with DTT produced a dose-dependent inhibition of specific [^{125}I]-angiotensin binding, with an IC_{50} of 1 mM and total loss of binding at 5–10 mM DTT. Inactivation of specific [^{125}I]-angiotensin II binding by DTT was temperature sensitive; the $t_{1/2}$ at 22° was 6 min compared with 30 min at 4°. A rapid inactivation rate was dependent on the presence of NaCl. In the presence of 120 mM NaCl the $t_{1/2}$ for inactivation by DTT was 6 min and 33 min in the absence of NaCl. Protection against DTT inactivation was obtained by preincubating membranes with unlabelled angiotensin II > angiotensin I > renin substrate while the dipeptide, Ileu–His was only effective in protecting the binding site at high concentrations (10 mM). Preincubations with DTT (1 mM) caused a 43% decrease in B_{max} from 217.0 ± 39.5 to 123.7 ± 30.9 fmol bound/mg protein while the K_D was not significantly affected.

A great deal of evidence has accumulated recently identifying essential sulphydryl groups and disulphide bridges on or in close proximity to, binding sites for a variety of ligands. Alkylation of the sulphydryl groups by *N*-ethyl maleimide (NEM) or reduction of the disulphide bridges by dithiothreitol (DTT) affects the properties of opiate receptors [1, 2] dopamine receptors [3, 4], acetylcholine receptors [5, 6], adrenoceptors [7, 8] and insulin receptors [8].

In the case of angiotensin II (AII) the results are more conflicting. Since DTT abolishes angiotensin-induced contractions of aortic strips [9] it has been suggested that a disulphide bridge is essential for the functional integrity of the vascular AII receptor. This proposal was supported by evidence that DTT decreased specific AII binding in rat mesenteric artery membranes [10] and bovine adrenal cortex membranes [11]. However, similar concentrations of DTT were reported to stimulate AII binding in urinary bladder and mesenteric artery membranes [12]. The reasons for such a discrepancy are not clear, although it has been suggested that other properties of DTT, such as its ability to inhibit proteases and its ion chelation effects, may in part be responsible for the disparity. Alternatively, since the physiological responses to AII are tissue dependent, the receptors may have different properties dependent on their tissue source. Since DTT has been routinely incorporated into incubation buffers in AII binding assays in order to reduce ligand degradation [13–15] it seems important to establish the sensitivity of AII receptors from a variety of tissue sources to the sulphydryl reagent.

The present study has examined the effects of DTT on specific [^{125}I]-AII binding to rat renal cortex epithelial membranes. These binding sites exhibit sodium-dependency [16] and are modulated by guanine nucleotides [17]. The study attempts to examine the relationships of an essential disulphide bridge with cationic and guanine nucleotide binding sites within the receptor complex.

* Author to whom correspondence should be sent.

MATERIALS AND METHODS

Monoiodinated [^{125}I]-angiotensin II (specific activity 1880 Ci/mmole) was obtained from NEN (Boston, MA, U.S.A.). Angiotensin and its analogues were purchased from Cambridge Research Biochemicals Ltd. (Cambridge, U.K.); DTT and all other chemicals were obtained from B.D.H. (Poole, U.K.).

Membrane preparation. Male Wistar rats, weighing approximately 250 g were killed by cervical dislocation and the kidneys removed immediately. The renal cortex was dissected on ice and homogenized (in a loose fitting homogenizer 0.43 mm clearance, Jencons) in 10% (w/v) isolation medium containing 250 mM sucrose, 10 mM triethanolamine HCl and 0.1 mM phenylmethylsulphonylfluoride (PMSF). Crude basolateral and brush-border membranes from cortical epithelial cells were prepared as described by Heidrich *et al.* [18] and the final membrane fraction used in ligand binding asays.

Radioligand binding assay. Membranes (15–25 μg protein) were incubated with [^{125}I]-AII (0.7–1.0 nM) for 5 min at 22° in 20 mM Tris–HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM Na_2EDTA, 0.1 mM PMSF and 0.2% BSA. Specific binding was defined by the addition of 1 μM unlabelled angiotensin II. Incubations were terminated by cooling and filtration through GF/B filters. Bound radioactivity was counted in a γ counter (Beckman Instruments). This method has been described in detail previously [16].

Treatment of the membranes with DTT was by preincubation in 20 mM Tris–HCl buffer, pH 7.4, in the presence or absence of NaCl. Following DTT preincubations at either 22° or on ice, aliquots of

[^{125}I]-AII and either unlabelled AII (1 μM) or vehicle were added simultaneously. The binding assay was allowed to proceed for a further 5 min at 22° before the reaction was terminated by cooling and filtration. In experiments where membranes were preincubated in the absence of NaCl, the salt was added at the same time as [^{125}I]-AII to give a final concentration of 120 mM.

In experiments where unlabelled peptides were used to protect the disulphide bridge from reduction by DTT, the following procedure was adopted. Membranes were incubated in 20 mM Tris–HCl buffer, pH 7.4, for 5 min at 22° in the presence of varying concentrations of unlabelled peptide before the addition of 10 mM DTT. After a 20 min incubation with DTT the tubes were placed on ice and diluted 100-fold with buffer. Membranes were collected by centrifugation (50,000 g for 10 min). This washing procedure was repeated twice and the final pellet resuspended in hypotonic isolation buffer, pH 7.6. This procedure removed the protecting drug and is based upon the method described by Freedman *et al.* [4]. Aliquots of the treated membrane preparation were assayed for specific [^{125}I]-AII binding as described above. The washing procedure reduced specific binding by approximately 33% and the total protein recovered was between 50 and 60% of the starting material.

Association and dissociation rates of specific [^{125}I]-AII binding were measured as described previously [16]. Association data was fitted to a pseudo first order rate equation and dissociation data to a first order rate equation. The resulting straight lines were obtained by computer assisted linear regression analysis. The ratio of the dissociation rate constant (k_{-1}) and the association rate constant (k_1) was used as an estimate of equilibrium constant, K_{eq}.

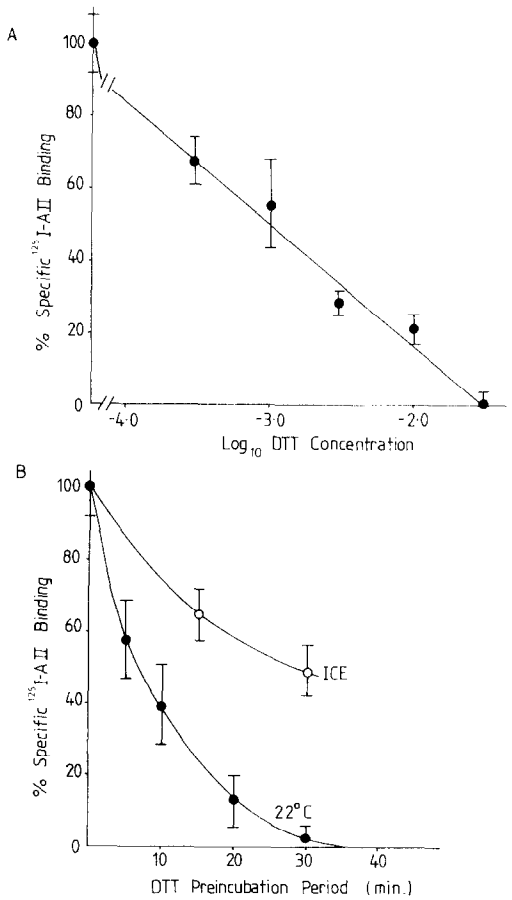

Fig. 1(A). Dose-dependent inhibition of specific [^{125}I]-AII binding by dithiothreitol. Renal cortex membranes were preincubated for 20 min at 22° with increasing concentrations of DTT. [^{125}I]-AII binding was estimated with 0.9 nM [^{125}I]-AII binding and 1 μM unlabelled AII to define nonspecific binding. Results are expressed as the % control binding (149.4 ± 12.1 fmole/mg protein) where membranes were preincubated in the absence of DTT. Each point represents the mean ± S.E.M. from a single representative experiment performed in quadruplicate. (B) Temperature sensitivity of DTT-induced inactivation of specific [^{125}I]-AII binding. Renal cortex membranes were preincubated with 10 mM DTT for increasing time intervals either at 22° (●) or on ice (○). Binding was assayed with 0.9 nM [^{125}I]-AII binding and 1 μM unlabelled AII to define non-specific binding. Results are expressed as % of zero time controls and each value represents the mean ± S.E.M. for quadruplicate incubations from a single experiment.

RESULTS

Characteristics of DTT inhibition

Specific binding to renal cortex membranes was reduced by DTT in a concentration-dependent manner (Fig. 1A). Following preincubation of membranes with increasing concentrations of DTT for 20 min at 22° a half-maximal inhibition was achieved with 1 mM DTT. Total inhibition of specific binding was routinely observed with 5–10 mM DTT. Inactivation of specific [^{125}I]-AII binding was temperature sensitive, preincubations conducted at 22° being fourfold faster than those at 4° (Fig. 1B). The rate of inactivation by DTT was also affected by the presence or absence of NaCl (Fig. 2). In the presence of 120 mM NaCl inactivation of specific [^{125}I]-AII binding was rapid with a $t_{1/2}$ of 6 min while in the absence of NaCl the inactivation rate was significantly reduced to a $t_{1/2}$ of 33 min.

The ability of AII to protect [^{125}I]-AII sites from disulphide group reduction by DTT was compared with that of renin substrate, angiotensin I (AI) and the fragment Ileu–His (this fragment is present in the 5 and 6 positions of the angiotensin molecule). The results (Table 1) confirm that AII was the most potent in protecting [^{125}I]-AII binding sites from DTT inactivation. Dose-dependent protection was obtained with all the peptides tested and half-maximal protection (EC_{50}) was calculated as the concentration of peptide needed to protect 50% of the binding sites from a maximal inhibitory concentration of DTT. The large differences (e.g. renin substrate) in absolute EC_{50} values may perhaps be partially attributable to the extra membrane washings and resuspensions necessary in the protection studies. Nevertheless the order of potency obtained from these protection studies was similar to that obtained from displacement studies [16], therefore suggesting that the DTT sensitive disulphide bridges

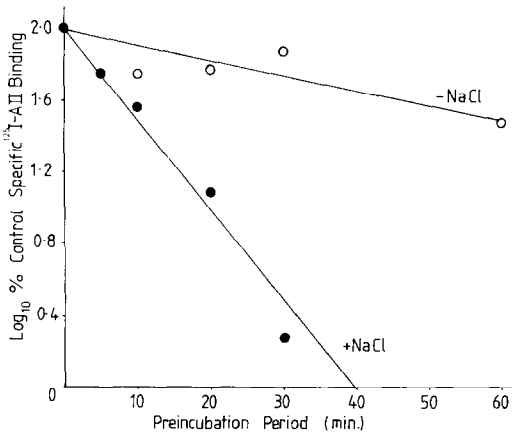

Fig. 2. Rate of inactivation of specific [125I]-AII binding by DTT in the presence and absence of NaCl. Renal membranes were preincubated with a maximally effective concentration of DTT (10 mM) for increasing periods of time at 22° in the presence or absence of NaCl (120 mM). Following preincubations conducted in the absence of NaCl the salt concentration was restored to 120 mM to enable assessment of specific [125I]-AII binding. Results are expressed as the $\log_{10}$% of control binding obtained from zero time controls. Each point represents the mean of at least 4 observations from a single representative experiment.

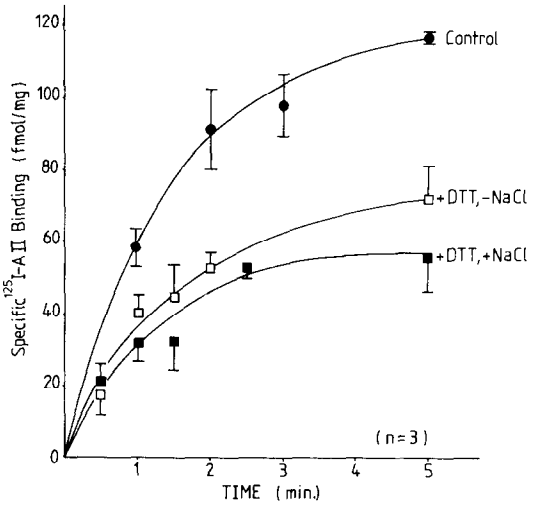

Fig. 3. Effect of DTT preincubation in the presence and absence of NaCl on the association rate of specific [125I]-AII binding. Renal cortex membranes were preincubated for 20 min at 22° with a 50% inhibitory concentration of DTT (1 mM) either in the presence (■) or absence of (□) of NaCl. Following preincubations in the absence of NaCl the final NaCl concentration was adjusted to 120 mM to allow maximal specific [125I]-AII binding. NaCl was added simultaneously with 0.9 nM [125I]-AII and 1 μM unlabelled AII to define specific binding. Binding assays were allowed to proceed for increasing periods of time before cooling and filtering through Whatman GF/B filters. Binding data was applied to a pseudo-first order rate equation and the slope of the resulting straight line (obtained by computer assisted linear regression) termed k_{obs}. Each point represents the mean ± S.E.M. from a single representative experiment (n values are shown in parenthesis in Table 2).

are located on or in close proximity to the [125I]-AII binding sites.

Kinetic alterations of [125I]-AII binding caused by DTT

The rates of [125I]-AII association and dissociation were measured following preincubation for 20 min at 20° in the presence or absence of 1 mM DTT, in the presence and absence of 120 mM NaCl. Preincubation of renal cortex epithelial membranes with 1 mM DTT routinely reduced the level of saturable specific [125I]-AII binding by approximately 50% in the presence of 120 mM NaCl and by 30% when NaCl was omitted. The observed rate at which specific binding reached equilibrium was not significantly altered by DTT, either in the presence or in the absence of NaCl (Fig. 3). The rate constant k_{obs} is therefore similar under all three conditions as shown in Table 2. Whether or not preincubations with DTT were conducted in the presence or absence of NaCl had little effect on the rate of dissociation of [125I]-AII from its binding site (Fig. 4). The resulting equilibrium constants, K_{eq} were therefore not significantly changed following preincubations with DTT. The kinetic constants obtained under all three conditions are shown in Table 2.

Table 1. Comparison of AII, AI, renin substrate and the dipeptide Ileu–His potencies in displacing specific [125]-AII binding and protecting AII binding sites from inactivation by DTT

Peptide	Protection EC_{50}	Displacement of specific [125I]-AII IC_{50}
Ileu^5–AII	100.0 nM	58.0 nM
Ileu^5–AI	7.3 μM	0.58 μM
Renin substrate	450.0 μM	2.34 μM
Ileu–His	3.5 mM	1.0 mM

Membranes previously incubated with at least four concentrations of unlabelled peptide were subjected to a further 20 min incubation with 10 mM DTT (120 mM NaCl was present at all times). Tubes were then diluted and membranes washed and binding assays performed as described in the Methods. The presence of DTT reduced binding to 29.2 ± 2.6% of control levels and this was used as the lower limit from which 50% protection (EC_{50}) was calculated for each peptide. Displacement data (IC_{50}) are shown for direct comparison and have been reported previously [16].

Although there was no significant change in the K_{eq}, the addition of DTT to renal cortex membranes resulted in a 43% reduction in the maximal binding capacity, B_{max} when saturation data was subjected to Scatchard analysis (Fig. 5). The maximum number of binding sites was reduced from 217 ± 39.5 fmole/mg protein under control conditions to 123.7 ± 30.9 fmole/mg protein following preincubations with DTT in the presence of NaCl. Using this form of analysis the affinity constants were 1.68 ± 0.54 nM following vehicle preincubation for 20 min at 22° and 1.14 ± 0.43 nM after 1 mM DTT preincubation. The discrepancy between the values obtained from these two methods of analysis probably reflect the lack of weighting in the computer fit analysis method used for the saturation data.

Table 2. Association and dissociation rate constants obtained under control conditions and following preincubation of renal cortex membranes with DTT in the presence and absence of sodium chloride

Condition	k_{obs} (sec^{-1})	k_{-1} (sec^{-1})	k_1 (M^{-1} sec^{-1})	K_{eq} (nM)
Control preincubation − DTT + 120 mM NaCl	$6.4 \pm 0.6 \times 10^{-3}$ (5)	$2.5 \pm 0.8 \times 10^{-3}$ (4)	$8.3 \pm 0.4 \times 10^{6}$	0.30 ± 0.02
Preincubation + 1 mM DTT + 120 mM NaCl	$8.9 \pm 0.7 \times 10^{-3}$ (3)	$1.3 \pm 0.2 \times 10^{-3}$ (3)	$11.4 \pm 1.7 \times 10^{6}$	0.13 ± 0.02
Preincubation + 1mM DTT − NaCl	$5.4 \pm 0.8 \times 10^{-3}$ (3)	$1.4 \pm 0.3 \times 10^{-3}$ (3)	$9.5 \pm 1.2 \times 10^{6}$	0.16 ± 0.02

Representative rate data shown in Fig. 3 and 4 yielded observed rate constants (k_{obs}) and dissociation rate constants (k_{-1}) respectively. The calculated association rate constant k_1 was obtained using the equation

$$k_1 = \frac{k_{obs} - k_{-1}}{[L]}$$

where [L] is the ligand concentration which varied from 0.7 to 1.0 nM. Numbers in parenthesis denote the number of experiments performed to give the final rate constant ± S.E.M.

Nucleotide modulation of DTT-inactivation of specific [^{125}I]-AII binding

The effect of guanine nucleotides on the DTT induced decrease in [^{125}I]-AII binding to rat renal cortex membranes was investigated. Results shown in Fig. 6 indicate that preincubation of renal membranes with 1 mM DTT reduced control binding by 42%. Preincubation of membranes with 1 μM Gpp(NH)p alone attenuated control binding by 30%. Addition of both DTT and Gpp(NH)p simultaneously, resulted in a significant reduction in specific ^{125}I-AII binding (78 ± 5%). This additive effect on binding appeared to be specific for guanine nucleotides. Ten μM GDP reduced specific binding by 30%, a similar value to that obtained with 1 μM GPP(NH)p. The presence of DTT and GDP together reduced binding by 68 ± 2%. In contrast preincubation of membranes with 10 μM ITP (which

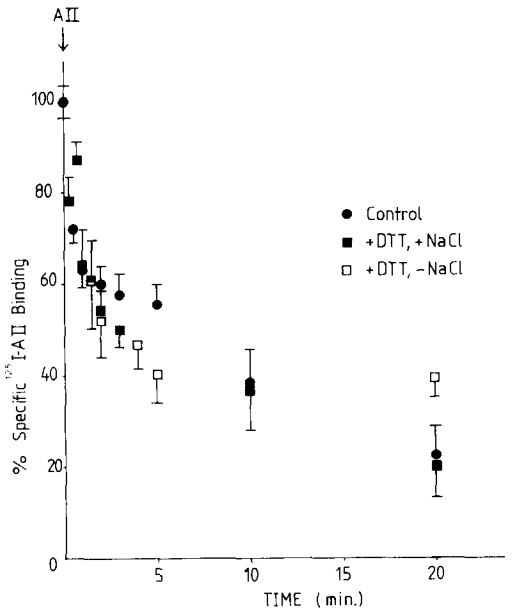

Fig. 4. Effect of DTT preincubation in the presence and absence of NaCl on the dissociation rate of specific [^{125}I]-AII binding. Kidney cortex membranes were preincubated for 20 min at 22° with DTT (1 mM) in the presence or absence of 120 mM NaCl. The NaCl concentration was restored in the latter case to 120 mM by addition of NaCl with 0.9 nM [^{125}I]-AII. After a further 5 min incubation 1 μM AII was added and tubes were filtered at varying time intervals after this addition. Binding data shown above was applied to the first order rate equation and the slope of the resulting straight lines taken to be k_{-1}. Each point represents the mean of three observations from a single representative experiment (N values are shown in parenthesis in Table 2).

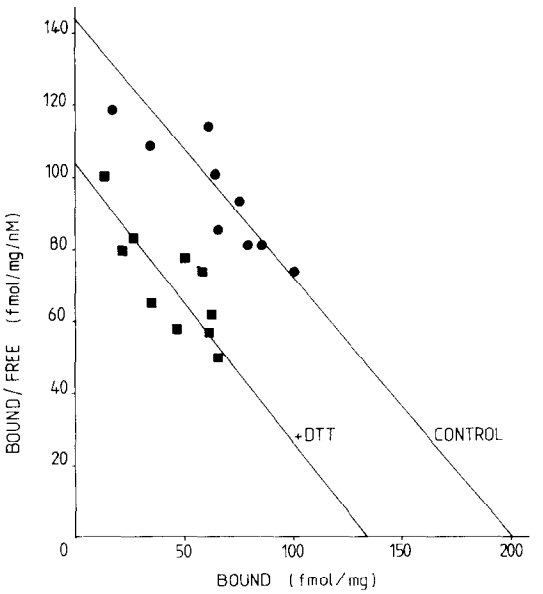

Fig. 5. Effect of 1 mM DTT preincubation on B_{max} and K_d for specific [^{125}I]-AII binding to renal cortex epithelial membranes. Renal membranes were preincubated for 20 min at 22° in the presence or absence of 1 mM DTT. 120 mM NaCl was present in all tubes at all times. Following preincubations increasing concentrations of [^{125}I]-AII and 1 μM AII (or vehicle) were added simultaneously and the binding incubation allowed to proceed for a further 5 min at 22° before termination by cooling and filtration. Binding data was highly reproducible and was therefore pooled from several experiments. Each point represents the mean from quadruplet determinations and errors were lower than 10%.

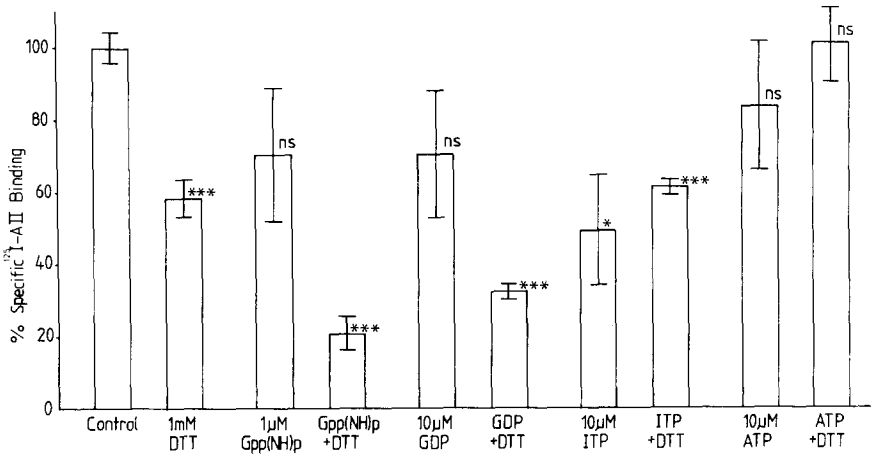

Fig. 6. Effect of various nucleotides on DTT inactivation of specific [125I]-AII binding. Kidney cortex membranes were preincubated for 20 min at 22° with 1 mM DTT, in the presence or absence of different nucleotides at the concentrations indicated. 120 mM NaCl was present at all times. Specific [125I]-AII binding was assayed by addition of 0.7–0.9 nM [125I]-AII and 1 μM AII or vehicle as described previously. Each point represents the mean ± 1 S.E.M. of 4 determinations from a single experiment and is expressed as % of control binding (169.5 ± 6.0 fmol/mg). * $P < 0.02$, ** $P < 0.01$, *** $P < 0.001$, unpaired Student's *t*-test.

reduced binding by 50%) and 1 mM DTT attenuated binding by only 40%. DTT induced decreases in specific binding were similarly reversed by the presence of 10 μM ATP. ATP alone reduced binding by 17% while in the presence of 1 mM DTT no reduction in specific binding was observed from control levels. The mechanisms underlying this guanine nucleotide additive inhibition are currently under investigation.

DISCUSSION

In agreement with previous studies of AII receptors in rat mesentery [10] and bovine adrenal cortex [11], specific [125I]-AII binding sites in a rat renal cortex epithelial cells were also sensitive to DTT. A dose-dependent reduction of specific binding was observed with DTT (IC_{50} 1 mM). This is in close agreement with the IC_{50} value obtained for DTT inhibition of [3H]-AII binding in bovine adrenal cortex membranes [11]. Rapid DTT inactivation of rat renal cortex [125I]-AII specific binding was temperature-sensitive and dependent on the presence of NaCl. DTT inactivation followed first-order kinetics in both the presence and absence of NaCl suggesting the existence of a single essential disulphide bridge. The half life of inactivation at 22° in the presence of 120 mM NaCl was 6 min compared with 33 min in the absence of NaCl. This is consistent with our previous findings that NaCl is necessary for specific [125I]-AII binding to rat renal cortex membranes [16], maximal binding being achieved with 120 mM NaCl. The effects of 120 mM NaCl in these experiments are specific for the cation and are not simply a reflection of increased ionic strength, since incubations containing 120 mM choline chloride, sucrose or varying Tris–HCl concentrations did not mimic the effects of NaCl (Cox *et al.*, unpublished results and [19]).

The results presented in this paper are similar in some characteristics to those obtained for sulphydryl group modification of [3H]-sulpiride binding in rat striatal membranes. [3H]-sulpiride binding is dependent on sodium for maximal specific binding and is inactivated by the alkylating agent NEM. Inactivation by NEM was, however, faster in the absence of sodium [4]. These results were explained as suggesting that an interaction of sodium with a cation-sensitive site close to the ligand binding site resulted in a conformational change in the membrane making the essential —SH group less accessible to NEM. By analogy the present study indicates that the presence of sodium may induce membrane-conformational changes resulting in the maximal exposure of a disulphide bridge present within or close to the AII binding site.

Preincubation of renal cortex membranes with unlabelled AII and to a lesser extent, renin substrate and AI prevented the inactivation of [125I]-AII binding by DTT. The dipeptide fragment Ileu–His was virtually inactive. These results support the contention that the DTT-sensitive disulphide bridge is on, or very near to the [125I]-AII binding site. The specificity of AII-protection against DTT also confirms that DTT is acting, in these experiments, as a disulphide-bridge reducing agent rather than as a chelating agent. Similar AII protection against DTT inactivation was reported for adrenal cortex specific [3H]-AII binding although the specificity of the protection has not been previously reported [11].

A marked (43%) reduction in the number of [125I]-AII binding sites was observed following preincubation of renal membranes with a half maximal concentration of DTT. Analysis of saturation data, as might be expected, failed to demonstrate any significant changes in the K_D under these conditions. This was confirmed by analysis of association and dissociation rate data. DTT did not affect the observed association rate constants whether NaCl

was present or not and the dissociation rates were also unaffected. Thus the K_{eq} was not altered by the DTT preincubations. There was a discrepancy between the absolute values obtained for K_d and K_{eq} in this study. This may be attributed, at least in part, to the lack of weighting in the computer analysis of saturation data, thus making it less sensitive for assessing small changes in affinity. Preincubation of renal cortex membranes with DTT significantly reduced the B_{max} of specific [^{125}I]-AII binding by 43%, a value which is similar to that obtained for DTT-sensitive adrenal cortex [^{3}H]-AII binding. In this case [11], following 20 min preincubation at 30° with 5 mM DTT, specific binding was reduced by 37% and no significant changes in K_d were observed.

It has been recently demonstrated that guanine nucleotides reduced specific [^{125}I]-AII binding with an increase in the affinity of the remaining sites [17]. The interactions of four different nucleotides at concentrations which maximally attenuate specific [^{125}I]-AII binding were investigated in the presence and absence of 1 mM DTT. Preincubation of renal cortex membranes with DTT and either GDP or Gpp(NH)p (the non-hydrolysable analogue of GTP) reduced specific binding in an additive fashion. This appeared to be selective for guanine nucleotides since ITP and ATP had no additional effect and prevented any further reduction in binding by DTT. Thus specific [^{125}I]-AII binding to renal cortex membranes is still attenuated by guanine nucleotides in the presence of DTT. The demonstration of such additive inhibition of binding indicates separate sites of action of DTT and guanine nucleotides.

Our results have shown the presence of an essential, DTT-sensitive disulphide bond within or extremely close to the [^{125}I]-AII binding site in rat renal cortex membranes. Interaction of the peptide with its receptor is modulated by sodium, perhaps allosterically through a separate membrane-associated cationic site. The presence of physiological concentrations of sodium allow for maximal specific [^{125}I]-AII binding and a more rapid inactivation of binding by DTT. On interaction with neighbouring cationic sensitive sites, sodium could produce a conformational change at the receptor site resulting in the exposure of an essential disulphide bond in the absence of the ligand, or allowing optimal binding in the presence of angiotensin.

REFERENCES

1. E. J. Simon and J. Groth, *Proc. natn. Acad. Sci. U.S.A.* **72**, 2404 (1975).
2. N. E. Larsen, D. Mullikin-Kilpatrick and A. J. Blume, *Molec. Pharmac.* **20**, 25 (1981).
3. E. T. Suen, E. Stefanini and Y. C. Clement-Cormier, *Biochem. biophys. Res. Comm.* **96**, 953 (1980).
4. S. B. Freedman, J. A. Poat and G. N. Woodruff, *J. Neurochem.* **38**, 1459 (1982).
5. R. S. Aronstam, L. G. Abood and W. Hoss, *Molec. Pharmac.* **14**, 575 (1978).
6. R. D. Schwartz and K. J. Kellar, *Molec. Pharmac.* **24**, 387 (1983).
7. G. Vanquelin, S. Bottari and A. D. Strosberg, *Molec. Pharmac.* **17**, 163 (1980).
8. L. Jarett and R. M. Smith, *Proc. natn. Acad. Sci. U.S.A.* **80**, 1023 (1983).
9. J. H. Fleisch, M. C. Krzan and E. Titus, *Circ. Res.* **33**, 284 (1973).
10. S. Gunther, M. A. Gimborne and R. W. Alexander, *Circ. Res.* **47**, 278 (1980).
11. R. S. L. Chang, V. J. Lotti and M. E. Keegan, *Biochem. Pharmac.* **31**, 1903 (1982).
12. G. Aguilera and K. J. Catt, *Circ. Res.* **49**, 751 (1981).
13. H. Glossmann, A. J. Baukal and K. J. Catt, *J. biol. Chem.* **249**, 825 (1974).
14. J. P. Bennett and S. H. Snyder, *J. biol. Chem.* **251**, 7423 (1976).
15. N. E. Sirett, A. S. McLean, J. J. Bray and J. I. Hubbard, *Brain Res.* **122**, 299 (1977).
16. H. M. Cox, K. A. Munday and J. A. Poat, *Br. J. Pharmac.* **79**, 63 (1983).
17. H. M. Cox, J. A. Poat and K. A. Munday, *Biochem. Pharmac.* **32**, 3601 (1983).
18. H. G. Heidrich, R. Kinne, E. Kinne-Saffran and K. Hannig, *J. Cell Biol.* **54**, 232 (1972).
19. H. M. Cox, PhD. Thesis, Southampton University (1983).