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Evidence of essential disulfide bonds in angiotensin II binding sites of rabbit hepatic membranes. Inactivation by dithiothreitol

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Radiolabelled angiotensin II binds to a single class of high-affinity binding sites on purified rabbit hepatic membranes. The binding is specific, reversible and saturable. Displacement studies using angiotensin and various analogs of angiotensin II disclosed a structure-activity profile similar to that found in physiologically relevant angiotensin II receptor sites. Treatment of membranes with the reducing agent, dithiothreitol, causes a significant decrease in the affinity of angiotensin II binding sites for the native ligand. This effect is mimicked by a 15-fold higher concentration of the monosulfhydryl derivative, 2-mercaptoethanol. Kinetic studies also indicated that dithiothreitol increases the rate of dissociation of bound ligand from the membrane without significantly affecting the association rate. In contrast, treatment of membranes with the metal chelators, ethylenediaminetetracetic acid (EDTA) and ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetracetic acid (EGTA), does not affect the binding of radiolabeled angiotensin II. Furthermore, dithiothreitol inhibited the binding of angiotensin II to a solubilized partially purified preparation of angiotensin II-binding protein from the same tissue and also increased the dissociation of bound angiotensin II. This indicates that the effect of the sulfhydryl reagents on the membrane binding sites is the result of a direct alteration of the binding sites rather than a gross modification of the structure of the membrane.

Introduction

Modification of the disulfide bonds or sulfhydryl groups present in a number of different types of receptor molecules frequently alters the binding of ligand to these specific cellular sites, thus affecting the initial step of hormone action. Dithiothreitol, a disulfide-reducing agent [1], affects ligand binding to different receptors in various ways. Treatment of nicotinic acetylcholine receptors of the central nervous system [2], β_1-adrenergic receptor in turkey erythrocytes [3] or TSH receptors in thyroid membranes [4] with dithiothreitol decreased the specific binding of agonist either by decreasing the receptor affinity [2,4] or by decreasing the number of high-affinity binding sites [3]. The same reagent caused a bimodal response of ^{125}I-labeled insulin binding to rat adipocyte plasma membranes [5]. At low concentrations of dithiothreitol, there was a dose dependent increase in binding, whereas at higher concentrations the binding decreased. Similar phenomena have been reported for other hormone receptors as well [6–9], and it was also suggested that one or several disulfide bonds were located adjacent to, or at the binding site of, the receptors [7].

Alteration of angiotensin II receptor activity was first suggested by the observation that dithiothreitol modified the angiotensin II-induced contractile response of isolated aortic strips [10]. More recently, the reagent was shown to decrease angiotensin II binding to its receptors in the particulate fraction of rat mesenteric artery [11] and

bovine adrenal cortex [12]. In adrenal cortical membranes, the effect of dithiothreitol, is readily reversible by the sulfhydryl oxidizing agent, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and similar effects were also observed on a solubilized receptor preparation [12]. We have recently shown that, in a digitonin solubilized partially purified angiotensin II-binding protein preparation from crude hepatic membranes [13], sulfhydryl reducing agents cause a marked increase in dissociation of angiotensin II from its complex with the angiotensin II binding protein [14]. In addition, the electrophoretic mobility of the affinity purified angiotensin II-binding protein was also significantly affected by these agents. In the present study, the mechanism of action of dithiothreitol on the angiotensin II receptors of purified rabbit liver membranes has been investigated by means of binding studies performed with radiolabeled ^{125}I-angiotensin II. We report that dithiothreitol diminished the affinity of the angiotensin II receptors significantly by increasing the rate of dissociation of bound ligand, whereas the receptor number was affected to a much lesser extent.

Experimental Procedure

Materials

Monoiodinated ^{125}I-angiotensin II (2200 Ci/mmol) was purchased from New England Nuclear. Unlabeled angiotensin II was from Bachem (Torrence, CA). Products of Sigma included Na_2EDTA, Tris, bovine serum albumin, dithiothreitol and 2,2′-thiodiethanol. [Sar^1,Leu^8]-angiotensin II, [Sar^1,Ile^8]-angiotensin II and angiotensin I were products of Vega Biochemicals (Tucson, AZ), and [Phe^3,Tyr^8]-angiotensin II was from Chemalog, Co. (Plainsfield, NJ).

Methods

Preparation of rabbit liver plasma membranes. Rabbit liver plasma membranes were prepared according to the method of Pohl [15] with slight modifications. Instead of 1 mM $NaHCO_3$ [16], 10 mM Tris-HCl (pH 7.4) was used, and the sucrose concentration in step 4 was increased to 45% (w/w) from 44% in the original procedure. The linear sucrose gradient centrifugation (steps 11–13) was performed in the same way. The membranes (1–3 mg protein/ml) were stored at −80°C for up to 5 months without loss of binding activity. Once thawed, the membranes were diluted to 0.5 mg/ml and used within 7–9 h.

Binding assay on membrane fraction. The purity of ^{125}I-angiotensin II purchased from New England Nuclear was evaluated by thin-layer chromatography as described earlier [13]. Binding experiments were done under conditions similar to those employed by Campanile et al. [16] for rat liver membranes. Reaction mixtures (total volume 0.15 ml) contained 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 0.2% heat-inactivated crystalline bovine serum albumin and 1 nM ^{125}I-angiotensin II unless otherwise specified. For saturation studies, unlabeled angiotensin II was added to ^{125}I-angiotensin II to achieve higher concentrations. The binding reaction was initiated by the addition of liver membranes (8–12 μg protein per tube) and continued for 1 h at 22°C, at which time 4 ml of ice-cold 20 mM Tris-HCl was added to the assay tubes to stop the reaction. ^{125}I-angiotensin II bound to protein was then separated from free ^{125}I-angiotensin II by vacuum filtration on 0.45 μm HA Millipore filters. Incubation tubes and filters were washed three times with 4 ml of 20 mM Tris-HCl and trapped radioactivity was measured in a gamma counter (counting efficiency 60%). Results are expressed as specific binding which is defined as the portion of total binding which can be displaced by an excess concentration (10 μM) of unlabeled ligand. Specific binding constituted 90–95% of total binding at a concentration of ^{125}I-angiotensin II of 1 nM.

Degradation of ^{125}I-angiotensin II by membrane fraction. The degradation of ^{125}I-angiotensin II by the membrane fraction was monitored essentially as described before [13]. The preparation was incubated with ^{125}I-angiotensin II as described above. After incubation, reaction mixtures were acidified and aliquots were spotted on a plastic backed cellulose thin-layer chromatography plate and developed in *tert*-butyl alcohol/3% ammonia (105 : 35, v/v). The plates were dried, cut into 0.5 cm strips, and the radioactivity was quantified in a gamma counter.

Solubilization and partial purification of angiotensin II binding protein. Membrane particles sedimenting between 1000 and 100 000 × g were ob-

tained from fresh rabbit liver homogenate and solubilized by treatment with the detergent, digitonin. Binding of ^{125}I-angiotensin II to the solubilized binding protein was assessed by radioactivity not adsorbed by a dextran-charcoal mixture, and critical results were confirmed by measurement of radioactivity in the void volume after gel filtration through a column of Sephadex G-50 [13]. Solubilized binding protein was purified by fractionation between 45 and 60% saturation with $(NH_4)_2SO_4$, gel filtration through Sepharose 6B, chromatography on SE-Sephadex C-50 and heat treatment. The detailed procedures have been published elsewhere [13,14].

Results

Characteristics of ^{125}I-angiotensin II binding to receptors of purified rabbit liver plasma membranes

The binding of ^{125}I-angiotensin II to purified liver membranes was specific and saturable. As shown in Fig. 1, specific binding reaches a plateau upon increasing the free concentration of the ligand. However, the nonspecific component of binding continued to increase linearly with increasing concentrations. Analysis of the saturation binding data according to Scatchard indicated the presence of a single class of binding sites with a dissociation constant (K_d) of 0.4 nM. The maximal number of binding sites was found to equal 2.6 pmol/mg of membrane protein. Saturation binding experiments were repeated on three different membrane preparations. The K_d values ranged between 0.3 and 0.6 nM, and the amount of binding sites ranged between 2.2 and 2.6 pmol/mg of protein. In contrast to these observations Companile et al. [16] reported the presence of two classes of binding sites in rat liver membranes. It is possible that this reflects a species difference.

The binding of angiotensin II at 22°C and pH 7.4 is rapid and reversible as measured by adding an excess of angiotensin II (10 μM) to an equilibrated mixture of ^{125}I-angiotensin II and the membrane preparation (described later). Binding reaches a maximum at pH 7.4 and is reduced to less than 50% below pH 6 and above pH 9 (Fig. 2). Increasing the concentration of free Mg^{2+} in the incubation increases binding 2–3-fold, and it remains the same between 1 and 20 mM, decreasing slightly above 50 mM (data not shown). This effect of Mg^{2+} on binding is similar to but less pronounced than that described by Campanile et al. [16] for rat liver membrane. No detectable degradation of ^{125}I-angiotensin II occurred during the incubation period.

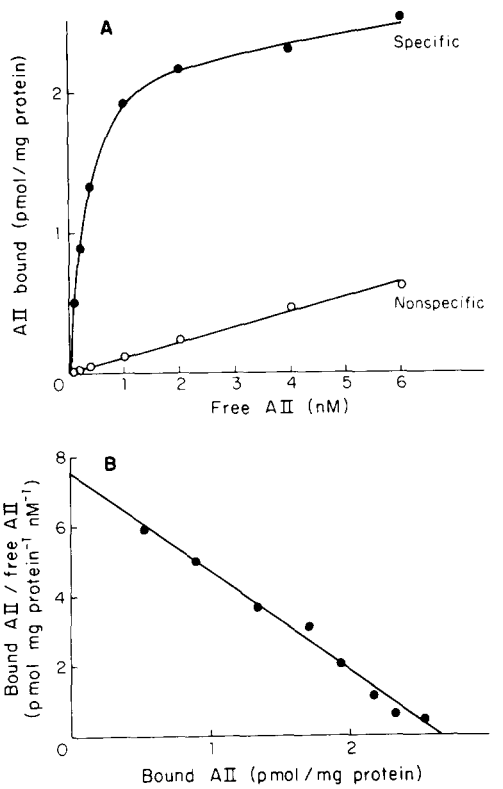

Fig. 1. Saturation binding of angiotensin II to rabbit liver membranes. (A) Rabbit liver membranes (9 μg) were incubated with increasing concentrations of tracer (0.1 to 6 nM) for 1 h at 22°C as described under Methods in the presence or absence of 10 μM unlabeled angiotensin II (AII). Bound angiotensin II was separated from free ligand by vacuum filtration. Specific binding (defined as the difference between total and nonspecific binding) is presented as a function of the concentration of free tracer. Each point represents the mean of duplicate determinations and this figure is representative of four such experiments utilizing three different preparations. ●——●, specific binding; ○——○, nonspecific binding. (B) Scatchard plot [17] of the saturation binding data. The line was drawn from the regression analysis of the data in (A) ($r = -0.98$). Free ligand is defined as the difference between the added ligand and the ligand bound to the membrane preparation. The dissociation constant K_d was 0.4 nM, and the number of binding sites B_{max} was 2.6 pmol/mg protein.

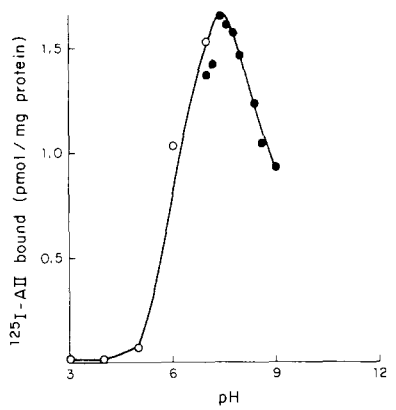

Fig. 2. pH dependence of angiotensin II (AII) binding to liver membranes. ^{125}I-angiotensin II (1 nM) was incubated with liver membranes (10 μg) as described under Methods, except that 50 mM Tris-HCl or citrate-phosphate buffer at the indicated pH was substituted for 20 mM Tris-HCl (pH 7.4). Total and nonspecific binding was determined at each pH value, and results are represented as specific binding per mg of protein. Data points represent pooled results from three different experiments, each one performed in duplicate, utilizing two different membrane preparations. O——O, Citrate-phosphate; ●——●, Tris-HCl.

Several analogs of angiotensin II were tested for their ability to inhibit the binding of ^{125}I-angiotensin II to the membrane preparation (Fig. 3).

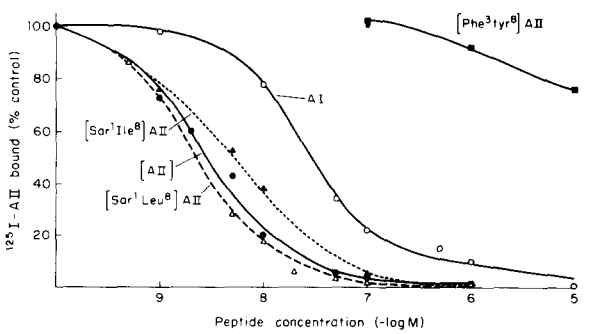

Fig. 3. Displacement of bound ^{125}I-angiotensin II (^{125}I-AII) by angiotensin analogs. Purified liver membranes (8–10 μg) were incubated with 1 nM ^{125}I-angiotensin II for 90 min at 22°C as described under Methods in the presence of varying concentrations of unlabeled angiotensin II or its analogs. Bound ligand was separated from free ligand by vacuum filtration. ^{125}I-angiotensin II binding in the presence of 10 μM unlabeled angiotensin II was subtracted from each value, and results were expressed as percentages of specific ^{125}I-angiotensin II bound in the absence of any unlabeled peptides. Data points represent the means of pooled data from three separate experiments with each assay performed in duplicate. Angiotensin I: Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-Leu^{10}.

The slope for binding inhibition by angiotensin I paralleled that observed for angiotensin II, but angiotensin I was 10–15-fold less potent an inhibitor than unlabeled angiotensin II. This was consistent with the low potency of angiotensin I to mimic the biological effects produced by angiotensin II in peripheral organs and in the central nervous system [18–29]. The two potent antagonists, [Sar^1,Ile^8]-angiotensin II and [Sar^1,Leu^8]-angiotensin II [29–31], were as effective as angiotensin II in inhibiting the binding of ^{125}I angiotensin II to the liver membrane preparation, while the weak agonist, [Phe^3,Tyr^8]-angiotensin II, inhibited poorly. All the displacement curves paralleled that for angiotensin II, suggesting that inhibition of binding was competitive.

Effect of sulfhydryl reagents on binding of ^{125}I-angiotensin II by liver membranes

The presence of disulfide bridge reducing agents, such as dithiothreitol or 2-mercaptoethanol, decreases the binding of ^{125}I-angiotensin II to its receptors in the liver plasma membrane preparation. The inactivation was determined as follows: Liver membranes were incubated with ^{125}I-angiotensin II and an increasing concentration of dithiothreitol or 2-mercaptoethanol, and the amount of ^{125}I-angiotensin II bound to the membranes was determined by filtration as described in Methods. The inactivation by the two thiol reagents was found to be dose dependent. As shown in Fig. 4,

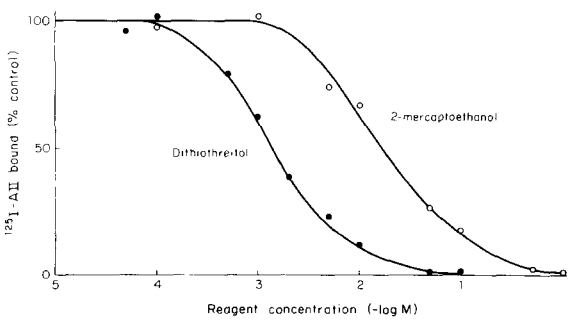

Fig. 4. Effect of sulfhydryl reagents on binding of ^{125}I-angiotensin II (^{125}I-AII) by liver membranes. Membranes were incubated with 1 nM of ^{125}I-angiotensin II for 1 h at 22°C in the presence of increasing concentrations of sulfhydryl derivatives. ●——●, Dithiothreitol; O——O, 2-mercaptoethanol. Control binding refers to the amount of ^{125}I-angiotensin II bound in the absence of any reagent.

the amount of ^{125}I-angiotensin II bound decreased progressively with with increasing concentrations of the thiol reagents. Under the given experimental conditions, half-maximal inhibition of ^{125}I-angiotensin II binding activity occurred at a dithiothreitol concentration of 1.2–1.5 mM. For 2-mercaptoethanol, the same inhibitory effect was observed only at concentrations exceeding 15 mM. These observations therefore indicate that dithiothreitol is approx. 10–15-fold more potent in inhibiting the angiotensin II receptors than its analog which contains only a single sulfhydryl group. At concentrations above 10 mM and 200 mM, respectively, of both agents, binding was less than 10%. In addition, 2,2'-thiodiethanol, a thioether, did not cause inactivation of ^{125}I-angiotensin II binding at a concentration as high as 200 mM. This effect of dithiothreitol is reversible since membranes incubated with the reagent and subsequently washed then binds angiotensin II in a manner similar to untreated membranes.

Change in angiotensin II receptor affinity in the presence of dithiothreitol

To analyze further the effect of dithiothreitol on angiotensin II receptors, liver membranes were incubated with an increasing concentration of ^{125}I-angiotensin II in the presence of 0.5 mM, 1 mM or 2 mM dithiothreitol. As shown in Fig. 5A, increasing concentrations of dithiothreitol inhibit ^{125}I-angiotensin II binding increasingly at all angiotensin II concentrations tested. Scatchard analysis of the binding data (Fig. 5B) indicated that in the presence of 0.5, 1 or 2 mM dithiothreitol, the affinity of angiotensin II receptors was 1.5, 2.5 and 3.3 nM, respectively, as compared to 0.6 nM in the control assay. With this progressive decrease in affinity, there is a corresponding decrease in the number of binding sites, but the effect is much less pronounced. The decrease in the number of binding sites (B_{max}) was 15%, 20% and 60%, respectively, with 0.5, 1 and 2 mM dithiothreitol, whereas the decrease in affinity was 250%, 400% and 600%, respectively.

Modification by dithiothreitol of dissociation rate of bound ^{125}I-angiotensin II

Liver membranes were incubated with ^{125}I-angiotensin II in the presence of different concentrations of dithiothreitol and allowed to reach equilibrium. Unlabeled angiotensin II was then added to the incubations to start dissociation. Fig. 6 shows that 50% of the bound ligand dissociates from the receptor in 6.3 h in the absence of any reducing agent, whereas in the presence of 2 mM reducing agent, the same amount of dissociation occurs in approx. 2 h.

Unlike dissociation, association of ^{125}I-angiotensin II to liver membranes is not significantly

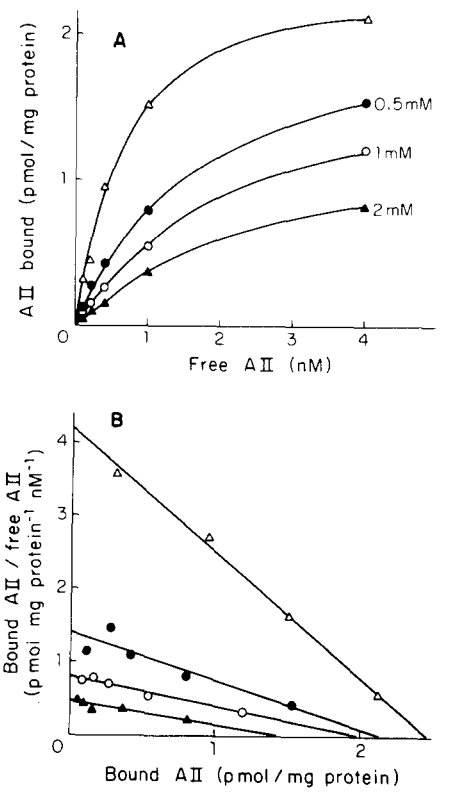

Fig. 5. Saturation binding of angiotensin II (AII) to rat liver membranes in the presence of dithiothreitol. (A) Membranes were incubated with 0.5 mM (●———●), 1 mM (○———○) or 2 mM (▲———▲) dithiothreitol and increasing concentrations of tracer (0.1–4 nM) for 1 h at 22°C. As a control, membranes were incubated with increasing concentrations of tracer only (△———△). Bound angiotensin II was determined by vacuum filtration as described earlier, and data is represented as specific binding per mg protein. (B) Scatchard plots of the saturation binding curves. Lines were drawn from the regression analysis of the data in (A). △———△, control (K_d, 0.6 and B_{max} 2.45 pmol/mg); ●———●, 0.5 mM dithiothreitol (K_d, 1.5 and B_{max} 2.1 pmol/mg); ○———○, 1 mM (K_d, 2.5 nM and B_{max} 1.9 pmol/mg); ▲———▲, 2 mM (K_d, 3.3 nM and B_{max} 1.5 pmol/mg).

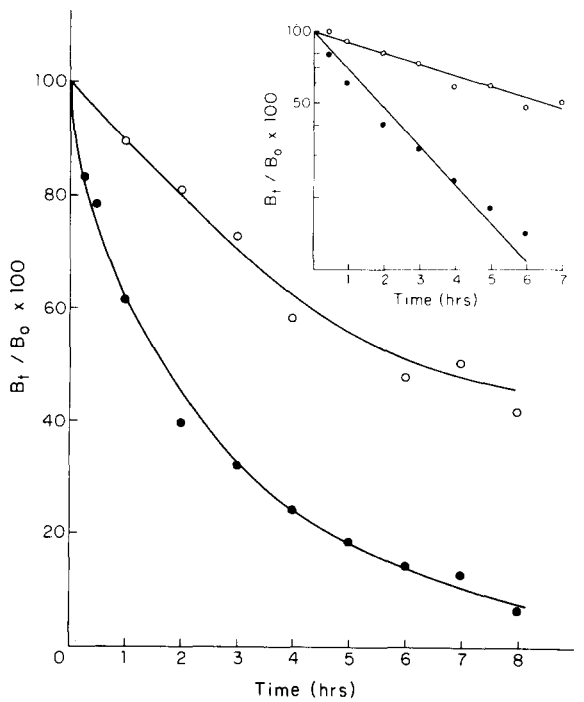

Fig. 6. Effect of dithiothreitol on time-course for dissociation of ^{125}I-angiotensin II from liver membranes. Liver membranes (150 μg) were incubated for 60 min at 22°C in the presence of 1 nM ^{125}I-angiotensin II in a final volume of 2.25 ml as described in Methods with (●) or without (○) 2 mM dithiothreitol. Then, 4.5 μl of 5 mM unlabeled angiotensin II were added, giving a final concentration of 10 μM in order to follow the dissociation kinetics of the labeled ligand. A 150 μl aliquot of the incubation mixture was withdrawn at the indicated time and filtered to separate bound and unbound hormone. B_0 and B_t represent the concentrations of bound angiotensin II at the start of dissociation and at any given time, respectively. Inset, semilogarithmic plot of the dissociation process.

affected by the presence of dithiothreitol up to a concentration of 2 mM.

Effect of dithiothreitol on ^{125}I-angiotensin II binding to digitonin solubilized, partially purified angiotensin II binding protein

Angiotensin II binding protein has been solubilized from a crude rabbit liver membrane preparation by treatment with digitonin and purified 300-fold by ammonium sulfate precipitation, gel filtration, ion exchange chromatography and heat treatment [13,14]. In the solubilized partially purified preparation, binding also occurs to a single class of sites. The availability of this solubilized preparation allows verification of whether dithiothreitol affects ^{125}I-angiotensin II binding by altering the overall structure of the membranes or by inactivating the receptors themselves. This preparation was incubated with ^{125}I-angiotensin II in the presence of various concentrations of dithiothreitol (0.1–100 mM) and the amount of ^{125}I-angiotensin II bound to the preparation was determined by the dextran-charcoal method [13].

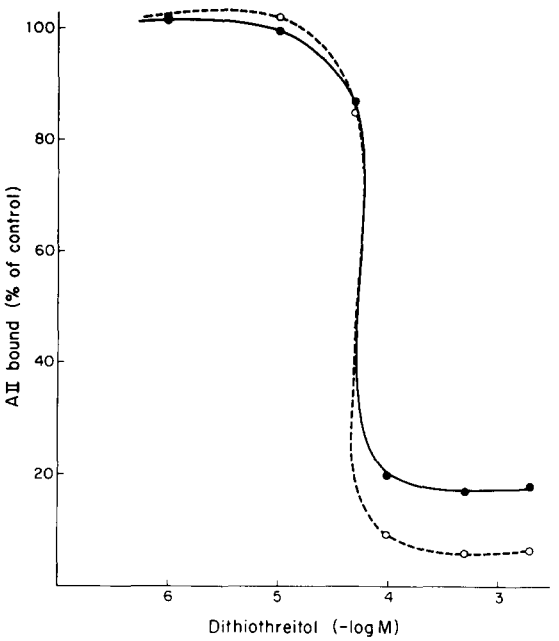

Fig. 7. Effect of dithiothreitol on binding of angiotensin II (AII) and its dissociation from solubilized preparation. ●——●, The digitonin solubilized partially purified angiotensin II binding protein preparation (1 mg) was incubated for 1 h at 22°C with 20 nM angiotensin II (19 nM unlabeled and 1 nM ^{125}I-angiotensin II), 20 mM Tris-HCl (pH 7.4), 4.5 mM Na_2EDTA, 158 mM NaCl, 0.1 mM *p*-chloromercuriphenylsulfonic acid, 0.25% Brij 99 and increasing concentrations of dithiothreitol in a final volume of 0.15 ml. Binding of angiotensin II to the solubilized preparation was assessed by the dextran-charcoal method [13]. Control binding refers to the amount of angiotensin II bound in the absence of dithiothreitol. ○——○, The digitonin solubilized preparation (10 mg) was incubated with angiotensin II as described above in a final volume of 1.5 ml. At 75 min, dithiothreitol was added to 150-μl aliquots of the incubation mixture to achieve the indicated concentrations. 15 min later, the remaining bound angiotensin II was assessed by the charcoal-dextran method. Control binding refers to the amount of angiotensin II bound with no added dithiothreitol.

Fig. 7 shows that binding of the ligand to this solubilized preparation is also inhibited by dithiothreitol in a dose dependent manner and that half-maximal inhibition of binding occurs at a 80 μM dithiothreitol concentration. Accordingly, the reducing agent affects ^{125}I-angiotensin II binding by direct alteration of the angiotensin II receptors. Furthermore, this inhibitory effect was not observed if 0.3 mM DTNB, an oxidizing agent, was included in the incubation. The oxidizing agent as such does not affect binding of ^{125}I-angiotensin II. On the other hand, when dissociation of bound angiotensin II from the solubilized preparation was followed at different concentrations of dithiothreitol (Fig. 7), it was observed that half maximal dissociation of bound ligand also occurred at 80 μM, and the two curves were virtually superimposable. This indicated that the inhibition of ligand binding by the sulfhydryl agent could probably be explained by its effect on the dissociation of bound ligand.

Finally, to rule out the possibility that dithiothreitol might be producing its effect by acting as a metal ion chelator, ^{125}I-angiotensin II binding to the solubilized protein was performed in the presence of various concentrations of potent metal chelating agents such as EDTA and EGTA; none of these agents mimicked the effect of dithiothreitol.

Discussion

In the present study we have investigated the role of disulfide bonds in the binding of angiotensin II to highly purified rabbit liver membranes. The presence of dithiothreitol during incubation of ^{125}I-angiotensin II with the membranes causes a dose dependent decrease in binding (Fig. 4). Several observations indicate that this decrease in angiotensin II binding is specifically a result of the cleavage of disulfide bonds. First, the inhibition of ^{125}I-angiotensin II binding activity by dithiothreitol is mimicked by another sulfhydryl derivative, 2-mercaptoethanol (Fig. 4). Moreover, the observation that dithiothreitol is 15-times more potent than 2-mercaptoethanol, which contains only one sulfhydryl group, is consistent with its higher reducing capability. [1]. Secondly, the inhibitory effect on solubilized angiotensin II binding protein is not observed if the oxidizing agent, DTNB, is present in the incubation with dithiothreitol, indicating that its action is mediated via reduction of disulfide bonds. Thirdly, the above data do not exclude the possibility that dithiothreitol may be producing its effects by acting as a metal-ion chelator. However, the observation that other potent metal-chelating agents such as EDTA and EGTA do not mimick its action favors the notion that reduction of disulfide bonds is the major effect of dithiothreitol, the consequence of which is a decrease in binding of ^{125}I-angiotensin II. Moreover, as similar effects of dithiothreitol are observed both on membrane bound and solubilized angiotensin II binding sites, the effect appears to result from a direct alteration of the binding sites by sulfhydryl agents, rather than from a gross modification of the structure of the membrane.

The angiotensin II binding sites of the liver plasma membranes behave as a homogeneous population with regard to the action of dithiothreitol since almost all of the binding sites could be inactivated by this agent (Fig. 4). Kinetic studies indicated that there was a 5-fold fall in the affinity of the binding sites for its native ligand (Fig. 5) in presence of 2 mM dithiothreitol, but the number of sites was affected to a much lesser extent. The decreased affinity was at least partly due to an increase in the rate of dissociation of the bound hormone. After completing this work, we became aware of a paper which reported the effect of dithiothreitol on the binding of ^{125}I-angiotensin II to rat liver membranes [32]. In contrast to our observation with rabbit hepatic membranes the number of high-affinity binding sites in rat liver membranes were reduced without significantly changing the affinity.

There is similar evidence that dithiothreitol alters angiotensin II binding in rat mesenteric artery [11] and in bovine adrenal cortex [12]. In the latter tissue, unlike in rabbit hepatic membranes, there was a reduction in the maximum number of membrane bound binding sites without a change in affinity. The above comparison indicates that angiotensin II receptors from different tissues are not equally affected by dithiothreitol. We have previously reported that an isolated purified angiotensin II-binding protein preparation ex-

hibited a slower electrophoretic mobility in its reduced rather than unreduced, form [14]. This observation, together with the reported decrease in binding of angiotensin II to its receptors in rabbit liver membranes and other tissues [11,12] in the presence of dithiothreitol, indicates that the disulfide bonds at or near the binding sites plays an important role in maintaining the proper binding configuration of this protein.

Acknowledgments

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