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Effect of Sodium Molybdate on Androgen Binding to Its Receptor from Shionogi Carcinoma 115

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ABSTRACT. The effects of molybdate on the binding characteristics of androgen receptor from the androgen-dependent mouse tumor (Shionogi carcinoma 115) with 17β-hydroxy-5α-androstan-3-one were studied by the charcoal adsorption method. The dissociation constant (K_d) was $(4.65\pm0.54)\times10^{-11}$ M in the presence of 10−12 mM sodium molybdate, which was one order of magnitude lower than the value without molybdate ($(4.54\pm0.72)\times10^{-10}$ M). Since the half life of the binding activity of unbound receptor was extended from 10 h to 75 h by adding molybdate, the effects of chaotropic reagents on binding were studied in the presence of molybdate. KCl (0.3−1.0 M) remarkably increased K_d without affecting the maximum binding capacity or the dissociation rate. KSCN (>0.1 M) had no effect on K_d but increased the dissociation rate, and while at concentrations at or above 0.5 M it completely inhibited the androgen binding to the cytosol receptor. Urea (0.5−2.0 M) increased K_d proportionally to the increase in the dissociation rate. K_d was also increased by 0.2−0.5 M guanidine, but the maximum binding capacity was reduced.

Key words : Androgen receptor — Sodium molybdate — Steroid binding — Shionogi carcinoma 115

The action of androgens in the target tissues is mediated by specific cytoplasmic binding protein(s) called the androgen receptor. The molecular interactions between androgens and their receptors are associated with a positive change in entropy[1,2] which is characteristic of hydrophobic interactions. We have observed recently that anions such as SCN^- and I^- severely interfered with the androgen binding to the receptor.[3] Others have reported that NaSCN increased the rates of association and dissociation between estrogen and its receptor without affecting the maximum binding capacity.[4] Valuable information for the chemical identity of androgen receptor can be obtained by the precise examinations of the effects of chaotropic salts on androgen-receptor interaction.

Molybdate has been used by several investigators to stabilize the unbound androgen receptor in cytosol.[5-7] However, they showed that molybdate had no effect on K_d. In this study we evaluate the effect of molybdate on the binding of androgen to the receptor from Shionogi carcinoma 115, and show that not only is the binding activity of unbound androgen receptor protected but also K_d is lowered from 10^{-10} M to 10^{-11} M by adding molybdate. We also evaluated the effects of KCl and other salts on the binding kinetics of

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androgen receptor because of the general usefulness in studying the physico-chemical properties of the receptor complex as well as for receptor purification.

MATERIALS AND METHODS

Chemicals

[1, 2, 4, 5, 6, 7-^{3}H] 17β-Hydroxy-5α-androstan-3-one (dihydrotestosterone) 114 Ci/mmole) was obtained from the Radiochemical Centre, Amersham. Non-radioactive dihydrotestosterone was purchased from the Sigma Chemical Co., St. Louis. Activated charcoal (Norit A) and dextran T-70 were obtained from the American Norit Co. and Pharmacia Fine Chemicals, respectively. Other chemicals were purchased from either the Nakarai Chemicals, Kyoto, or the Wako Pure Chemicals, Osaka.

Preparation of cytosol

The androgen-dependent Shionogi carcinoma 115 has been maintained by serial subcutaneous transplantation into 6- to 8-week-old male DS mice as described previously.[8] The tumor used in the experiments was obtained from mice castrated 48 h earlier. The tumors were dissected free of subcutaneous tissues and necrotic debris, and minced in chilled Buffer A (10 mM Tris-HCl, 0.1 mM EDTA, and 2 mM dithiothreitol, pH 7.4). The minced tumor fragments were homogenized in 4-5 volumes of the same buffer using a Polytron PT-10 homogenizer at setting 7 for 30 sec at 0°C. The homogenate was centrifuged at 2°C for 60 min at 100,000 x *g* to obtain clear supernatant (cytosol). Where indicated, 1 M Na_2MoO_4 was added to the homogenate to give a final concentration of 10-12 mM, and Buffer A containing 10 mM Na_2MoO_4 (Buffer B) was used instead of Buffer A. This concentration of sodium molybdate was chosen on the basis of data presented in Figure 2. Protein concentrations in the cytosol were determined by the modified method[9] of Lowry *et al.*[10] with crystalline bovine albumin as a standard.

Measurement of androgen binding to cytosol receptor

Androgen binding was measured by the charcoal adsorption assay[1] using [^{3}H]dihydrotestosterone as ligand. Binding specificity was checked in parallel trials by adding a 100-fold molar excess of unlabeled dihydrotestosterone. Nonspecific binding of [^{3}H]dihydrotestosterone was usually less than 30% of the total binding, and was substracted from the total binding in all cases. Stock solutions of KCl, KSCN, urea, and guanidine HCl in Buffer A (4-6-times higher concentrations) were added to each incubation mixture to give final concentrations shown in results.

Dissociation rate

The tumor cytosol was preincubated at 0°C for 20 h with 5 nM [^{3}H] dihydrotestosterone to saturate the receptor with labeled androgen, and a 1,000-fold excess of unlabeled dihydrotestosterone was added to a portion of the mixture. The mixture was further incubated at 0°C for 1-5 h, and the receptor-bound [^{3}H]dihydrotestosterone was measured by the charcoal adsorption assay. Controls without addition of dihydrotestosterone were used to estimate the stability of androgen-receptor complex in parallel. First order rate constants were calculated from regressions of percent of receptor-bound [^{2}H]dihydrotestosterone as a function of time in semilogarithmic plot.

Determination of radioactivity

Radioactivity was determined by liquid scintillation spectrometry in a Searle M-III scintillation spectrophotometer (model 6880) as described.[1] Counting efficiency was approximately 35% for tritium.

RESULTS

The stabilizing effect of molybdate on the binding activity of unbound androgen receptor was examined by using the cytosol of Shionogi carcinoma 115 prepared in Buffer A. Rapid inactivation of the receptor at 15°C ($t\frac{1}{2}$ = 1 h) was prevented by the inclusion of 10 mM sodium molybdate during incubation (Fig. 1). Molybdate stabilized the binding activity of unbound receptor

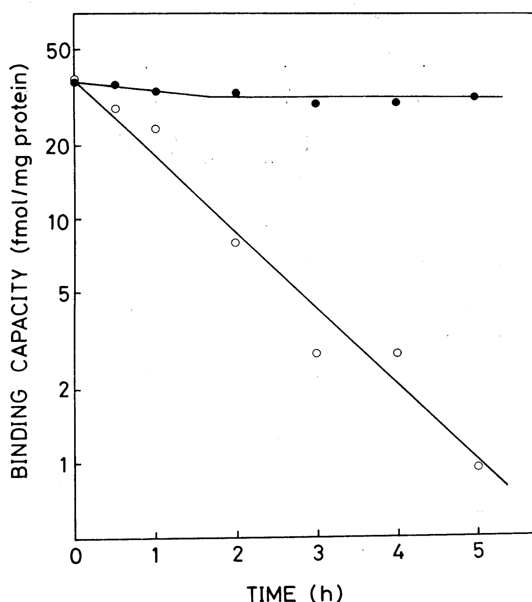

Fig. 1. Effect of molybdate on the stability of unbound androgen receptor at 15°C. Tumor cytosol was prepared in Buffer A, and incubated at 15°C in the presence (●) or absence (○) at 10 mM sodium molybdate. At various times, aliquots were removed and incubated for 15–20 h at 0°C with 20 nM [^{3}H] dihydrotestosterone in the presence or absence of 10 mM sodium molybdate respectively. Specific binding capacity was assayed by the charcoal adsorption method.

in a concentration-dependent manner (Fig. 2). The optimum concentration was 10–30 mM. These results correspond with those reported for the androgen receptor in the rat prostate[5] and the mouse kidney[7] and for the glucocorticoid receptor in the rat liver.[11] Inactivation at 0°C ($t\frac{1}{2}$ = 10 h) over longer intervals up to 140 h was examined (Fig. 3). Molybdate (10 mM) completely prevented loss of binding activity up to 20 h. Thereafter binding activity gradually decreased ($t\frac{1}{2}$ = 75 h). Furthermore no loss of binding activity of unbound receptor in the tumor cytosol was detectable at -80°C in the presence of molybdate for at least 30 days. At -20°C, however, a considerable decrease

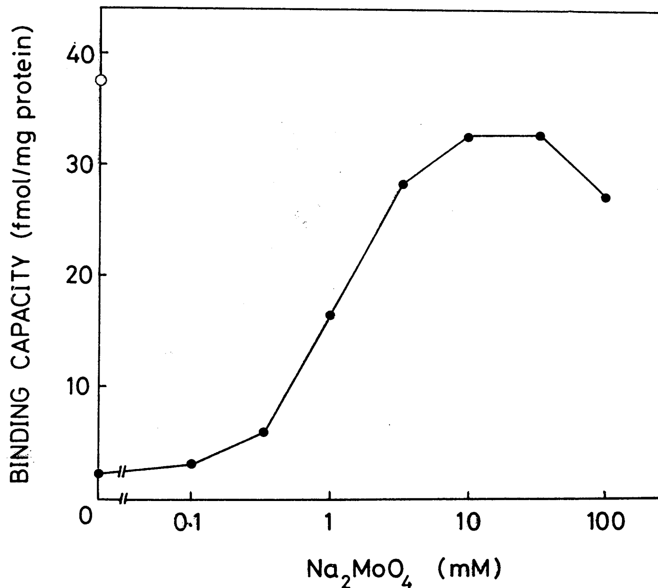

Fig. 2. Effect of several concentrations of molybdate on the stability of unbound androgen receptor at 15°C. Tumor cytosol prepared in Buffer A was incubated for 3 h at 15°C in the presence of various concentrations of sodium molybdate or buffer. Specific binding capacity was measured in the presence of sodium molybdate at the same concentration as specified for the initial 3 hr incubation. ○, binding capacity of zero molybdate control; ●, binding capacity samples assayed after 3-h incubation in the indicated concentration of molybdate.

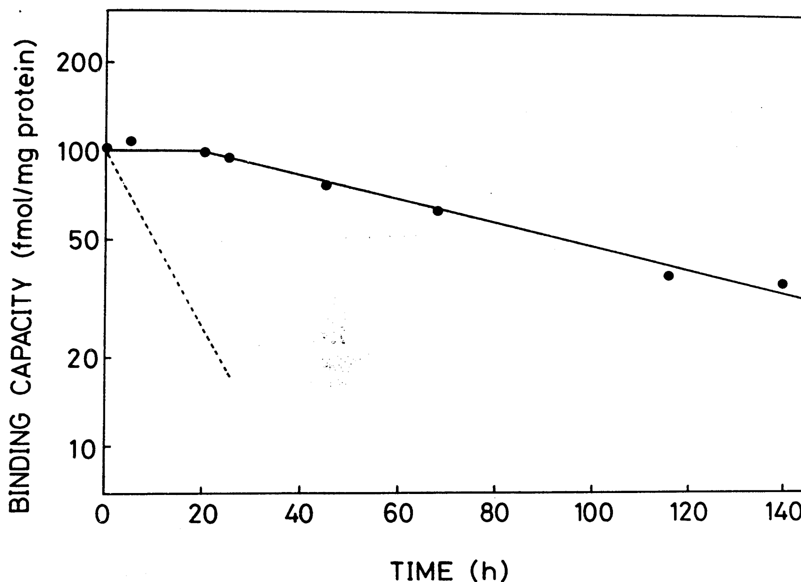

Fig. 3. Effect of molybdate on the stability of unbound receptor at 0°C. Tumor cytosol prepared in Buffer B was incubated at 0°C (●). At various times, aliquots were assayed for binding activity in the presence of 10 mM molybdate with 20 nM [^{3}H] dihydrotestosterone for 5 h at 0°C. Specific binding capacity was determined by the charcoal adsorption method. ----, inactivation curve in the absence of molybdate.[1]

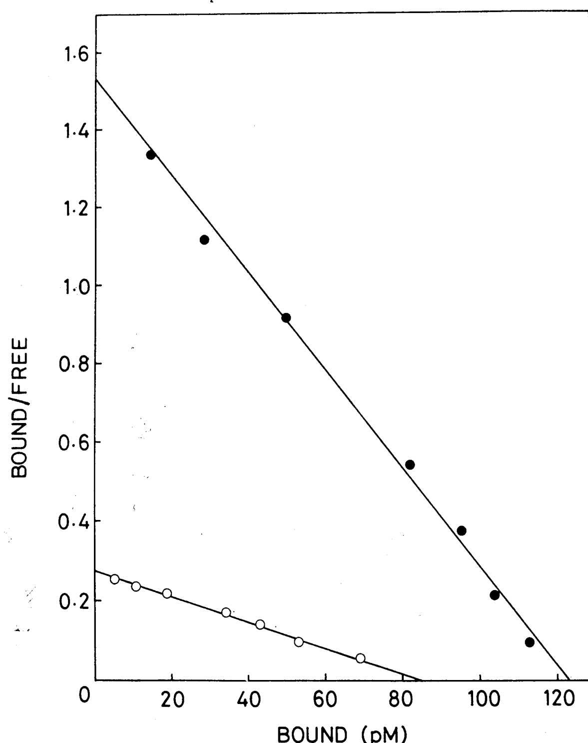

Fig. 4. Saturation analysis of androgen receptor in the presence or absence of molybdate. Tumor cytosol prepared in Buffer A (○) or Buffer B (●) was incubated for 16 h at 0°C with 0.1–2.0 nM [^{3}H]dihydrotestosterone. The samples were assayed for specific binding by the charcoal adsorption method, and the data presented by Scatchard analysis.

of the binding activity occurred even in the presence of molybdate and at nearly the same rate as observed at 0°C (data not shown).

When saturation binding analysis was performed by incubating the cytosol receptor with 0.1–2.0 nM [^{3}H]dihydrotestosterone for 16 h at 0°C in the presence or absence of molybdate, the dissociation constants calculated by the Scatchard plot were significantly different (Fig. 4). The apparent dissociation constants were $(4.52\pm0.72)\times10^{-10}$ M (mean±S.E., n=11) in the absence of molybdate and $(4.65\pm0.54)\times10^{-11}$M (n=8) in the presence of 10–12 mM molybdate. The latter values were almost the same as those previously estimated by the kinetic analysis of androgen-receptor interaction in the cytosol of this tumor.[1] Therefore, the remaining experiments were carried out in the presence of Buffer B instead of Buffer A during homogenization and incubation.

Since a slow dissociation rate would be expected to seriously affect the estimation of the association constant under the usual conditions of saturation analysis, the incubation time required for reliable estimates was determined experimentally. When the tumor cytosol was incubated with 0.2–2.0 nM [^{3}H] dihydrotestosterone at 0°C in the presence of 10 mM molybdate, the apparent association constant calculated by the Scatchard analysis was not constant but rather varied with the incubation time in a non-linear fashion (Fig. 5). Early

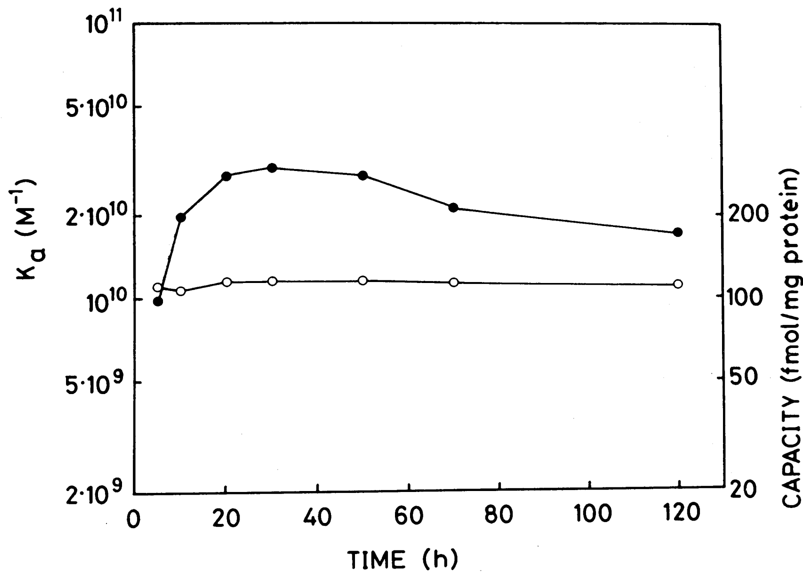

Fig. 5. Effect of incubation time on the association constant and maximum binding capacity measured by saturation analysis. Tumor cytosol was prepared in Buffer B and incubated at 0°C with 0.2–2.0 nM [^{3}H]dihydrotestosterone for various times. The association constant (●) and maximum binding capacity (○) of androgen receptor were estimated by Scatchard analysis of the binding data.

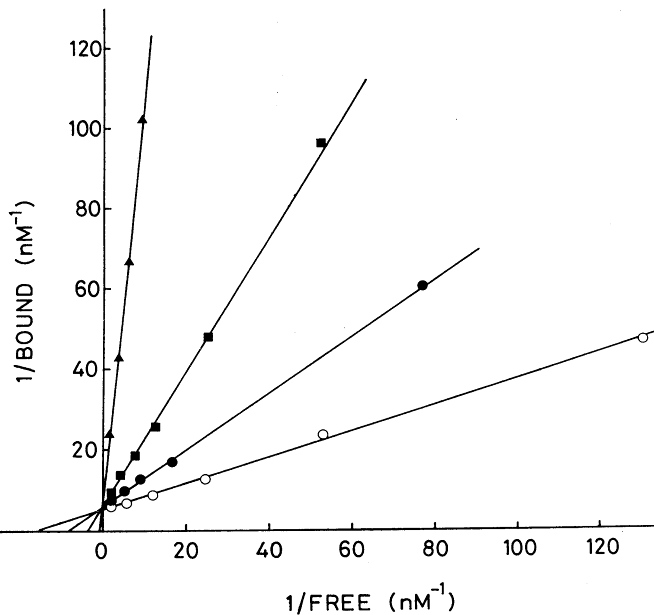

Fig. 6. Effect of KCl on the equilibrium binding of androgen to the receptor. Tumor cytosol prepared in Buffer B was incubated with 0.1–2.0 nM [^{3}H] dihydrotestosterone for 20 h at 0°C in the presence of 0.3 M MCl (●), 0.5 M KCl (■), 1.0 M MCl (▲), or buffer (○). The samples were assayed for specific binding by the charcoal adsorption method. Double reciprocal plots of the specific binding vs. unbound [^{3}H]dihydrotestosterone are shown.

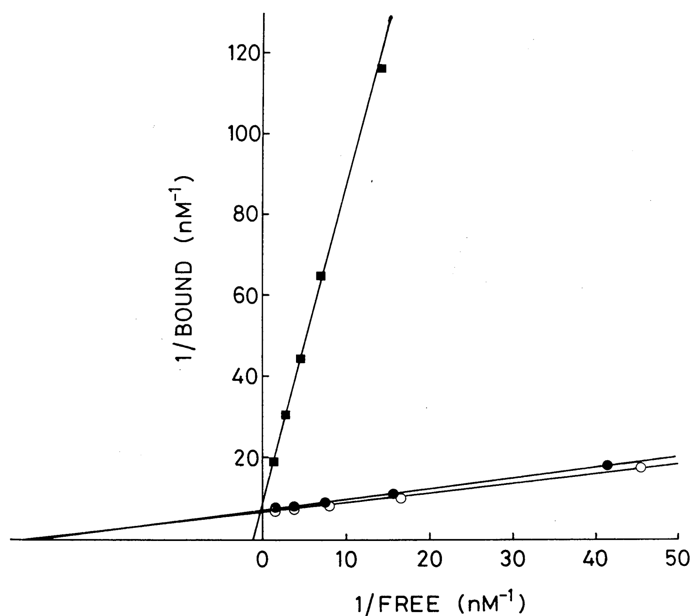

Fig. 7. Effect of KSCN on the equilibrium binding of androgen to the receptor. Tumor cytosol was incubated with 0.1–2.0 nM [^{3}H]dihydrotestosterone for 24 h at 0°C in the presence of 0.1 M KSCN (●), 0.3 M KSCN (■), or buffer (○). Others were the same as in Fig. 6.

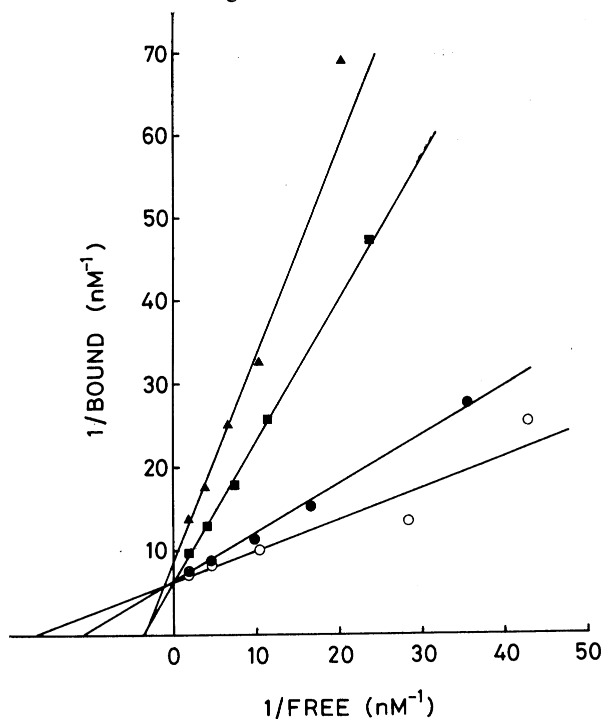

Fig. 8. Effect of urea and guanidine on the equilibrium binding of androgen to the receptor. Tumor cytosol was incubated with 0.1–2.0 nM [^{3}H]dihydrotestostrone for 24 h at 0°C in the Presence of 0.5 M urea (●), 1.0 M urea (■), 0.2 M guanidine HCl (▲), or buffer (○). Others were the same as in Fig. 6.

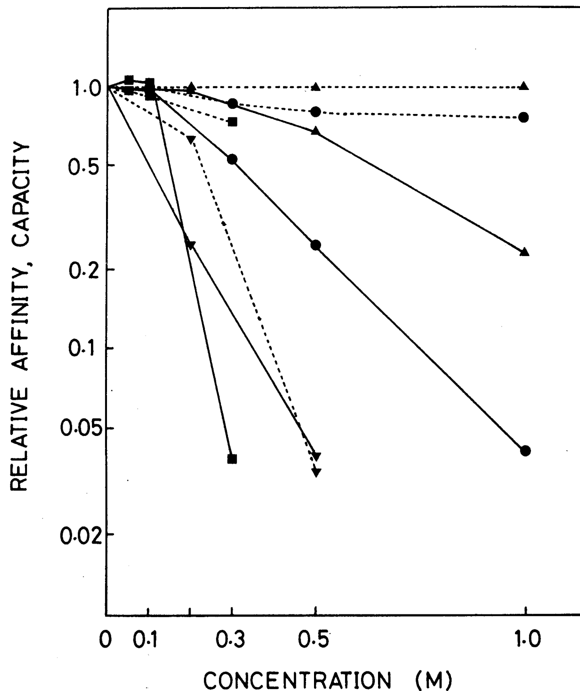

Fig. 9. Effects of KCl, KSCN, urea, and guanidine on the binding affinity of capacity of androgen receptor. The binding affinity (equilibrium association constant) and the maximum binding capacity were estimated by the saturation analysis of the binding data obtained in the presence of several concentrations of KCl (●), KSCN (■), urea (▲), and guanidine HCl (▼) under the conditions as described in Figs. 6–8. The relative binding affinity (——) and binding capacity (- - - -) were calculated as a ratio of the value in the presence of these compounds of that of the control (buffer).

times less than 20 h showed an increase with time. Incubation times longer than 30 h at 0°C resulted in some loss of binding capacity of unbound receptor (Fig. 3). Since the loss of binding capacity during incubation was more pronounced at lower concentrations of ligand, it seriously affected estimates of the association constant, although the apparent number of maximum binding sites determined by Scatchard analysis was almost unchanged (Fig. 5). Therefore, we employed a 20–30 h incubation time at 0°C as the optimum condition.

To investigate the effect of KCl on the binding of androgen to the receptor, the tumor cytosol was incubated in the presence or absence of KCl in addition to molybdate under the conditions described above. The binding data were analyzed by the double reciprocal plot (Fig. 6). Although 0.1 M KCl had no effect on the androgen binding to the receptor, the apparent association constant was decreased by the addition of 0.3–1.0 M KCl accompanying a slight but significant decrease of maximum binding capacity (see also Fig. 9).

KSCN also had a marked inhibitory effect on the androgen binding activity at a concentration of 0.3 M or above (Fig. 7). The binding affinity and the maximum binding capacity were almost unchanged at 0.01–0.1 M KSCN, but 0.3 M KSCN caused a drastic decrease of the binding affinity. At concen-

trations of 0.3 M or higher, the binding activity was completely inhibited.

Urea and guanidine reduced the binding affinity at concentrations of 0.5 M and 0.2 M, respectively (Fig. 8). However, the inhibition pattern of individual reagent differed.

Relative binding affinities in the presence of KCl, KSCN, urea, or guanidine are summarized in Fig. 9. All of these compounds reduced the binding affinity, but they differed in their ability to alter the binding capacity. KSCN and guanidine caused a steep decline of the affinity in the same concentration range that decreased maximum binding capacity, while KCl and urea showed a gradual decrease of the affinity with slight or no reduction of the binding capacity up to 1.0 M.

Effects of these compounds on the dissociation rate were examined next. When KCl, KSCN, and urea were added to the incubation mixture at concentrations ineffective to reduce the maximum binding capacity, changes of the dissociation rate by these compounds did not correlate with the effects of the equilibrium dissociation constant (Fig. 10). KCl, although increasing the dissociation constant at 0.3–0.5 M, slightly decreased the dissociation rate, and urea produced an increase of dissociation rate in proportion to the increase of dissociation constant. On the other hand, 0.03–0.1 M KSCN showed a marked increase of dissociation rate without influencing the equilibrium dissociation constant.

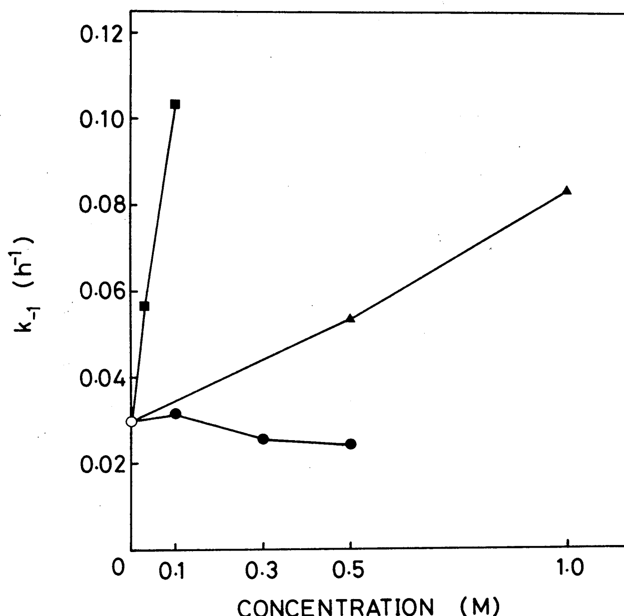

Fig. 10. Effects of KCl, KSCN, and urea on the dissociation rate of androgen–receptor interaction. Tumor cytosol prepared in Buffer B was incubated for 20 h at 0°C with 5 nM [^{3}H]dihydrotestosterone. KCl (●), KSCN (■), and urea (▲) were added to aliquots of the mixture together with a 1,000-fold excess of unlabeled dihydrotestosterone. Each mixture was further incubated at 0°C for 1–5 h, and the residual specific binding of [^{3}H]dihydrotestosterone was assayed by the charcoal adsorption methed. First order rate constant (K_{-1}) was calculated from regression of percent of the specific binding as a function of time.

DISCUSSION

Inactivation of unbound androgen receptor at low temperatures was prevented by 10–30 mM sodium molybdate (Figs. 1–3). Although the stabilizing effect of molybdate is common to the androgen receptors, there still remain major discrepancies between our results and those reported previously by Gaubert *et al.*,[5] Wright *et al.*,[7] and Traish *et al.*[6] These investigators showed that the binding constant for androgen interaction with receptor was not influenced by the presence of molybdate when the equilibrium binding data were analyzed by the Scatchard plot. On the contrary, our results clearly indicate that the dissociation constant determined in the presence of molybdate was one order of magnitude lower than that obtained in the absence of molybdate (Fig. 4), and is in agreement with the value estimated by earlier kinetic analysis.[1] Since the inactivation rate of unbound receptor is higher than the dissociation rate in the absence of molybdate, the dissociation constant previously calculated by the Scatchard analysis is overestimated. When inactivation of unbound receptor is prevented by molybdate and the reaction allow to come to equilibrium (20–30 h), a reliable estimate is obtained by saturation analysis. Thus, the estimate of the dissociation constant in the presence of molybdate can be considered the more accurate one.

Another possibility about the discrepancy of the effect of molybdate is that we used native androgen, dihydrotestosterone, as a ligand while Gaubert *et al.*,[5] Wright *et al.*,[5] and Traish *et al.*[6] used synthetic androgen, R1881. Rates of the interaction with androgen receptor are higher in R1881 than in dihydrotestosterone. Therefore, they could not detect the effect of molybdate on the dissociation constant by Scatchard analysis.

KCl decreased the association constant of the androgen–receptor interaction (Figs. 6, 9). Since the dissociation rate was unchanged in 0.1–0.5 M KCl (Fig. 10), decreased binding affinity with KCl should be proportional to the decrease of the association rate. KCl is known to inhibit estrogen binding to uterine cytosol[12,13] and glucocorticoid binding to its receptor.[14,15] Unlike the reports on estrogen receptor[13] and glucocorticoid receptor,[15] the maximum binding capacity of the cytosol androgen receptor was not seriously reduced by 0.1–1.0 M KCl (Fig. 9). With respect to estrogen receptor of uterine cytosol, 0.4 M KCl was demonstrated to increase the dissociation rate at 0–10°C.[12,16] A similar result has been reported for glucocorticoid–receptor complex.[15] These results are in contrast to our result in the presence of molybdate (Fig. 10). The apparent decrease of the binding affinity of androgen receptor by KCl is, therefore, mediated through a mechanism different from the effect of KCl on the receptor binding of estrogen and glucocorticoid.

In the presence of molybdate, KSCN caused a marked inhibition of the androgen binding to the cytosol receptor at concentrations of 0.3 M or higher. The effect of 0.3 M KSCN on the relative affinity was equivalent to that of 1.0 M KCl (Fig. 9), where the apparent association constant decreased to less than one-tenth of the control accompanying a slight reduction of binding capacity. The dissociation constant was not affected by 0.03–0.1 M KSCN, whereas the dissociation rate was remarkably increased (Fig. 10). The increased dissociation rate by 0.03–0.1 M KSCN may be accompanied by the equivalent

increase of the association rate. The similar effect of SCN^- was reported for the estrogen-receptor interaction in calf uterus,[4] but the effectiveness of SCN^- in increasing the rates of association and dissociation of estrogen binding to the receptor was lower than that of androgen binding. Furthermore, 0.5 M NaSCN had no effect on the maximum binding capacity of estrogen receptor.[4] On the other hand, our results show that 0.5 M KSCN completely abolished the binding activity of androgen receptor. Therefore, the androgen receptor is more susceptible to SCN^- than the estrogen receptor.

We also investigated the effect of moderate concentrations of urea and guanidine on androgen binding to its cytosol receptor in order to assess the importance of hydrogen bonding in the process. Our interpretations of these data assume that we are working with unpurified receptor. Therefore, they must be viewed as tentative until purification has been achieved.

Urea increased the dissociation constant of androgen-receptor interaction without affecting the binding capacity (Figs. 8, 9). Since the dissociation rate was increased proportionally to the increase of the dissociation constant (Fig. 10), the association rate seemed to be unchanged by 0.5–1.0 M urea. Urea is known to rupture hydrogen bonding. Therefore, conformational change induced by the impairment of intramolecular hydrogen bond could promote the dissociation of androgen from the receptor.

Inhibition of androgen binding to the receptor by guanidine was somewhat different from those of the reagents discussed above. Guanidine reduced the maximum binding capacity as well as the binding affinity (Fig. 9). Although guanidine has a water structure-breaking property similar to that of SCN^-,[17] the effect of guanidine on the binding capacity was more evident than that of KSCN. Reduction of the affinity to one-twentieth of the control by guanidine was accompanied with an equivalent decrease of binding capacity, while KSCN-induced reduction of the affinity to the same extent was not accompanied by such a severe decrease (Fig. 9).

Acknowledgments

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