Vol. 108, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1982 Pages 822-827

INTERACTION OF SERINE PROTEASE INHIBITORS AND SUBSTRATES WITH HUMAN UTERINE ESTROGEN RECEPTOR.

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Received June 5, 1982

The human uterine estrogen receptor has a site which regulates estrogen binding and which structurally resembles the substrate binding site of chymotrypsin. The hormone binding capacity and the affinity of the receptor is decreased in the presence of 4 mM serine protease inhibitors tosyl-lysine chloromethyl ketone and diisopropylfluorophosphate and the protease substrates tryptophan methyl ester and toluene sulphonyl-arginine methyl ester. The protease inhibitors tosylamide-phenylethyl-chloromethyl ketone and phenyl methyl sulphonyl fluoride caused an increase in the binding capacity whereas the affinity was decreased.

INTRODUCTION

The human uterine cytoplasmic 9.0 S estrogen receptor is degraded to a 4.2 S fragment by an endogenous protease (1,2). This degradation, which is associated with the loss of the *in vitro* nuclear binding activity of the receptor, is blocked by the serine protease inhibitor diisopropylfluorophosphate (1,3). The estradiol binding capacity of the estrogen receptor is however decreased in the presence of DFP (1). Either the 9.0 S estrogen receptor form has a lower binding capacity than the proteolytic fragment, or DFP competes with estradiol for the binding to the hormone binding site. There are no experimental data which would support the latter concept. It has however been suggested that all steroid hormone receptors, including rat α-fetoprotein, would contain a binding site for some protease

ABBREVIATIONS USED: PMSF - phenylmethyl sulphonylfluoride; TAME - toluenesulphonyl-arginine methyl ester; TLCK - tosyl-lysine chloromethyl ketone; TME - tryptophan methyl ester; TPCK - tosyl-amide-phenylethyl-chloromethyl ketone; DFP - diisopropylfluorophosphate.

0006-291X/82/180822-06$01.00/0

inhibitors as well as for protease substrates (4,5). The presented data show that also the human uterine estrogen receptor has a binding site which recognizes serine protease inhibitors and substrates. This site may play a role in the regulation of estrogen binding.

MATERIALS AND METHODS

The buffer used was 40 mM Tris/HCl-1mM dithiothreitol, pH 7.4 (TD buffer).

Preparation of uterine cytosol was performed as described previously (3). The protease substrates and inhibitors were included into the homogenization buffer (4 mM). The protein content of the cytosols as measured by the method of Lowry et al. (6) were 7-9 mg/ml.

Saturation binding analysis and association rate studies were performed as described earlier (7). The equilibrium dissociation constants and the binding capacities were calculated according to the method of Scatchard (8). Density-gradient centrifugations were carried out as described earlier (7). DNA-cellulose was prepared as described by Alberts & Herrick (9). The DNA content as measured by the method of Burton (10) was 135 µg per ml cellulose. DNA binding studies were performed as batch assays by incubating aliquots of labelled cytosol with DNA-cellulose (1ml packed cellulose) for 1 h at 4 °C under continuous agitation. After centrifugation the pelleted DNA-cellulose was washed three times with TD buffer. The bound receptors were then extracted with TD + 0.6 M KCl for 16 h at room temperature. The radioactivity was monitored as described earlier (7).

2,4,6,7-^{3}H-Estradiol (sp. radioactivity 92.0 Ci/mmol) was purchased from New England Nuclear Corporation. The purity was checked by t.l.c. on silica gel using a solvent system consisting of benzene-ethylacetate-acetic acid (60:40:0.5, by vol.). Estradiol-17β, PMSF,TPLCK,TME and TAME were purchased from Sigma Chemical Co.. DFP was obtained from Fluka AG.

RESULTS AND DISCUSSION

TAME, TME, DFP and TLCK caused a decrease in the total number of estradiol binding sites whereas TPCK and PMSF induced an increase in the binding capacity (Fig.1). Baker et al. (4) found that TPCK is the most effective inhibitor of the binding of aldosterone, dexamethasone, dihydrotestosterone and progesterone to their respective receptors and that PMSF is the most effective inhibitor of estradiol binding to rat α-fetoprotein. They added the protease inhibitors and substrates to the receptor incubation mixture simultaneously with the hormone. In contrast, we found

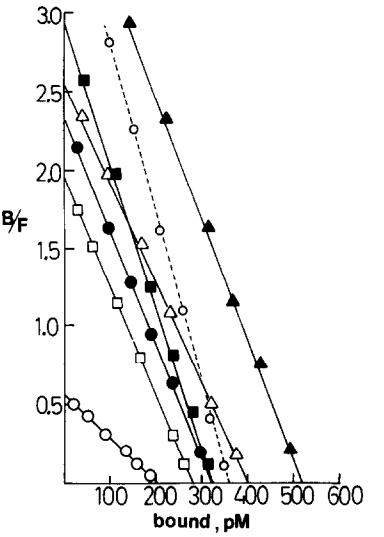

Figure 1. Scatchard plots of ^{3}H-estradiol binding to uterine estrogen receptors in the presence and absence of 4 mM protease inhibitors and substrates.
Control (o---o), TLCK (o), TPCK (△), TME (□), TAME (●) PMSF (▲), DFP (■).

that when the substrates or inhibitors were added to the receptor preparation at the same time as the hormone no change in the estrogen binding was observed (data not shown). The equilibrium dissociation constant of estrogen binding in the human uterine cytosol is very low (Kd = 0.07 nM) as compared to the affinities of the other steroid receptors for their respective ligands (5,11, 12,13). The difference between the present results and those of Baker et al. (4) may partly be ascribed to this difference in the ligand binding affinities of the receptors. Furthermore, the interaction of the steroid with its specific binding site may result in such conformational changes in the receptor protein that will affect the binding of other ligands to other sites on the protein. Therefore, quite different results are obtained if for instance an allosteric inhibitor is added before or simultaneously with the ligand to the binding protein preparation.

Competitive inhibition of binding is indicated by decreased affinity and unaltered binding sites in the presence of the com-

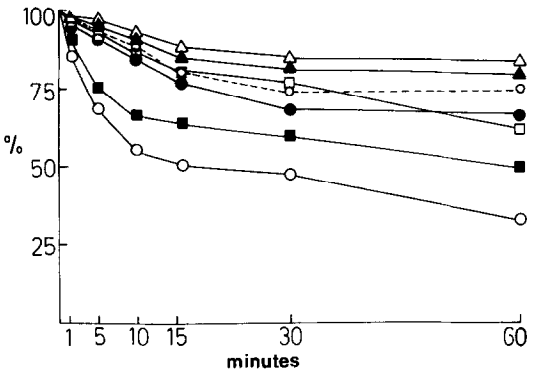

Figure 2. Stability of the ^{3}H-estradiol receptor complex at 37 °C in the presence and absence of 4 mM protease inhibitors or protease substrates. Aliquots of cytosol were equilibrated with 5 nM tritiated ligand for 4 h at 0 °C in the presence and absence of protease inhibitors and substrates. The incubation was continued at 37 °C and samples were removed at the time intervals indicated. After adsorption of unbound ligand to charcoal-dextran the aliquots were counted for radioactivity. Control (o---o), TLCK (o), TPCK (△), TME (□), TAME (●), PMSF (▲), DFP (■).

petitor. The binding of dexamethasone to the glucocorticoid receptor and of estrone to rat α-fetoprotein is competitively inhibited by TME (5,11). The presented data suggest, that TAME, TME, TLCK and DFP inhibit the estradiol binding rather by an allosteric than by a competitive mechanism. The protease inhibitors PMSF and TPCK act through a positive co-operative mechanism which induces an increase in the total binding (Fig. 1).

We also tested how the substrates and inhibitors affected the stability of the estrogen receptor complex at 37 °C. As is shown in Figure 2 the same compounds that caused a decrease in the binding capacity caused also a decrease in the stability of the hormone receptor complex. PMSF and TPCK had a slight stabilizing effect on the binding (Fig. 2).

It was also of interest to study the association velocity in the presence and absence of the different compounds. The data are presented in Table 1. The high association rate in the presence of DFP confirms our previous observation (3). TPCK and TAME had no effect on the association whereas TLCK and TME caused an

Table 1. Equilibrium dissociation constants (Kd) obtained from Scatchard plots and association rate constants (k_{+1}) determined from the linear portions of second order plots. The dissociation rate constants (k_{-1}) were calculated from $Kd = k_{-1}/k_{+1}$.

Competitor	Kd (nM)	$k_{+1} \times 10^{-5}$ ($M^{-1}s^{-1}$)	$k_{-1} \times 10^{5}$ (s^{-1})
-	0.07	3.05	2.07
TLCK	0.39	4.58	17.80
TPCK	0.15	3.12	4.55
DFP	0.11	5.55	6.04
PMSF	0.13	2.08	2.62
TME	0.15	4.58	6.68
TAME	0.15	3.19	4.65

increase in the association velocity. The lowest rate constant was obtained in the presence of PMSF. The calculated dissociation rate constants (Table 1) also show clearly the difference in the effect of the tested compounds.

The DNA binding capacity of the estrogen receptor was greatest in the presence of DFP (Table 2). In an earlier report from our laboratory we have described how also the *in vitro* nuclear binding of the uterine estrogen receptor is greatly increased in the

Table 2. Sedimentation values (S) and DNA-binding activities of the uterine estrogen receptor in the presence and absence of protease inhibitors and substrates.

Competitor	Sedimentation value (S)	DNA-binding (% of added activity)
-	4.2	4.3
TLCK	4.2	4.0
TPCK	4.2	2.1
DFP	4.2; 9.0	11.3
PMSF	4.2	3.6
TME	4.2	2.9
TAME	4.2	2.5

presence of DFP (3). This effect of DFP is associated with its ability to block the proteolytical degradation of the cytoplasmic 9.0 S receptor form. This seems to be valid also for the DNA binding of the estrogen receptor since the 9.0 S receptor form could be detected only in the presence of DFP (Table 2).

The present paper shows that protease inhibitors and substrates interact with the human uterine estrogen receptor at a site which causes alterations in the estrogen binding. It has been shown that binding of progesterone to its meroreceptor is inhibited by protease inhibitors and substrates (4). This observation points to the conclusion that the locus on the progesterone receptor which binds the compounds is close to the steroid binding site. The presented data do not give information about the spatial relationship between the two binding sites on the estrogen receptor. The locus which resembles the binding site on serine enzymes may however play an important role in the regulation of receptor function.

ACKNOWLEDGMENTS. This work was supported by Tor, Pentti and Joe Borgs Foundation and by Paulo Foundation.

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