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Inhibition by Protease Inhibitors of Binding of Adrenal and Sex Steroid Hormones

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Binding of steroid hormones is inhibited by protease inhibitors and substrates. The protease inhibitors phenylmethyl sulphonylfluoride, tosyl-lysine chloromethyl ketone, and tosylamide-phenylethyl-chloromethyl ketone and the protease substrates tosyl arginine methyl ester and tryptophan methyl ester eliminate specific binding of aldosterone, dexamethasone, dihydrotestosterone, estrogen, and progesterone to their respective receptors. These protease inhibitors and substrates also inhibit binding of progesterone to the 20,000 molecular weight mero-receptor formed from the progesterone receptor in chick oviduct. The binding of estradiol to rat alpha-fetoprotein is inhibited by the protease inhibitors and substrates but not by tryptophan or tryptophan amide, indicating the importance of an ester structure in the inhibition of steroid binding. Our results suggest that all steroid hormone receptors have a site with both common structural features and a role in the regulation of steroid hormone binding.

Key words: **aldosterone, dexamethasone, dihydrotestosterone, estrogen, progesterone, steroid hormone receptor**

The binding of steroid hormones by their specific intracellular receptors is a crucial initial step in steroid hormone action. We previously reported that the specific binding of deoxycorticosterone (DOC) to its receptor in Madin Darby Canine Kidney (MDCK) cells was eliminated by protease inhibitors and substrates [1]. We now show that certain protease inhibitors and substrates also inhibit binding of glucocorticoid and sex steroid hormones to their specific receptors. We infer that the structure of all steroid receptors contains a site that has both common structural features and a role in the regulation of steroid hormone binding.

Abbreviations used: PMSF – phenylmethyl sulphonylfluoride; TAME – p-toluenesulphonyl-arginine methyl ester; TLCK – tosyl-lysine chloromethyl ketone; TME – tryptophan methyl ester; TPCK – tosylamide-phenylethyl-chloromethyl ketone; AFP – alpha-fetoprotein; TES – N-tris (hydroxymethyl) methyl-2-amino-ethanesulfonic acid; Tris – tris-(hydroxymethyl)-aminoethane; EDTA – ethylene diamine tetraacetic acid.

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MATERIALS AND METHODS

Reagents

PMSF, TPCK, TLCK, L-TAME, D-TME, L-TME, and unlabeled steroids were purchased from Sigma Chemical Co; D-TAME, from Vega-Fox Chemical Co. Tritiated aldosterone (82 Ci/mmole), dexamethasone (33 Ci/mmole), dihydrotestosterone (190 Ci/mmole), 17 β estradiol (152 Ci/mmole), and progesterone (114 Ci/mmole) were purchased from New England Nuclear Corporation.

Receptor Sources

Aldosterone receptor was obtained from kidneys of adrenalectomized rats. Dexamethasone receptor was obtained from rat hepatoma tissue culture cells (HTC) cells [2, 3] that were a gift from Dr. Keith Yamamoto. Dihydrotestosterone receptor was obtained from Shionogi carcinoma S115 cells [4] that were a gift from Dr. Walter Desmond. The source of rat alpha-fetoprotein (AFP) was serum from buffalo rats with Morris Hepatoma 7777. The AFP was a gift from Dr. Stewart Sell. Progesterone receptor was obtained from the oviducts of female Rhode Island Red chicks that had received diethylstilbestrol for 20 days [5, 6].

Cell Culture

HTC and S115 cells were cultured in Dulbecco-Vogt modified Eagle's medium with 10% fetal calf serum on 100 mm Falcon tissue culture dishes. Under these conditions, HTC and S115 cells grow attached to the dish.

Preparation of Cytoplasmic Supernatant Fractions

All procedures were done at 0–4°, unless otherwise stated. In our initial experiments, we used a homogenization buffer consisting of 10 mM Tris pH 7.6 + 2 mM EDTA + 7 mM 2-mercaptoethanol. In later experiments we used a homogenization buffer consisting of 20 mM TES pH 7.9 + 2 mM EDTA + 7 mM 2-mercaptoethanol, because TES has better buffering capacity below pH 8 and a lower temperature-dependent pH variation than Tris [7]. Similar results for steroid binding to cytosol were obtained with both buffers. Rat kidney and chick oviduct were homogenized with a Brinkman Polytron, centrifuged 10 min at 10,000 × g, and then centrifuged 1 h at 190,000 × g. HTC cells and S115 cells were homogenized with a dounce homogenizer and centrifuged 1 h at 190,000 × g.

Hormone Binding Assay

Steroid binding assays were carried out on the cytosol and AFP in glass tubes on ice that contained tritiated steroid alone or with protease inhibitors or substrates or a 200- to 500-fold concentration of unlabeled steroid. For progesterone binding experiments chick oviduct cytosol was diluted 25-fold with homogenization buffer containing 5 mg/ml ovalbumin. Bound steroid was separated from unbound steroid using a dextran-coated charcoal technique similar to that described by Baxter and Tomkins [2] and Rosseau et al [3]. We added 0.1 ml of 300 mg/ml charcoal + 30 mg/ml dextran in the homogenizing buffer to 0.9 ml cytosol. After the solution was vigorously mixed and incubated for 4 min, the tubes were centrifuged at 6,000 × g for 10 min. The radioactivity in 0.5 ml aliquots of the supernatant was determined in a liquid scintillation cocktail containing Omnifluor, Triton X-100, and toluene. We also separated bound steroid from unbound steroid by filtration on

Sephadex G-25 columns (1 X 16 cm). One-half ml cytosol equilibrated with steroid or steroid and inhibitors was loaded on the column equilibrated with homogenizing buffer with 0.3 M KCl, and eluted with this buffer.

Radioactivity bound in the presence of a 200- to 500-fold excess of unlabeled steroid was assumed to be nonspecifically bound steroid. This was subtracted from radioactivity bound with the labeled steroid alone to determine the specifically bound steroid.

RESULTS AND DISCUSSION

We find that the protease inhibitors PMSF, TLCK, and TPCK inhibited the binding of aldosterone to rat kidney cytosol (Table I), dexamethasone to HTC cell cytosol (Table I), dihydrotestosterone to S115 cell cytosol (Table II), estrogen to rat alpha-fetoprotein (Table II), and progesterone to chick oviduct cytosol (Table III). We also find that the protease substrates TME and TAME inhibit binding of these steroids to their receptors (Table IV). These results extend our previous findings that these protease inhibitors and substrates could inhibit binding of deoxycorticosterone to its receptor in MDCK cells [1] to other classes of steroid hormone receptors.

Tentative generalizations may be made from the results presented in Tables I–IV. TPCK is the best inhibitor of aldosterone, dexamethasone, dihydrotestosterone, and progesterone binding. A concentration of about 25 micromolar TPCK inhibits binding of these steroids by about 50%. PMSF and TLCK are about 50 to 100 times less effective in inhibiting steroid binding. In contrast, PMSF is the most effective and TPCK and TLCK less effective in inhibiting estrogen binding to rat AFP.

TABLE I. Inhibition of Steroid Binding by Protease Inhibitors

Steroid	Inhibitor	Inhibitor concentration	Specifically bound steroid 10^{-13} moles/ml	% Inhibition
Aldosterone	–	–	2.1	–
	TPCK	2.5×10^{-4}M	0.21	90
		2.5×10^{-5}M	0.97	54
		5.0×10^{-6}M	1.70	19
	TLCK	1.0×10^{-3}M	0.17	92
		2.5×10^{-4}M	1.51	28
	PMSF	1.0×10^{-3}M	0.92	56
		2.5×10^{-4}M	1.39	34
Dexamethasone	–	–	1.9	–
	TPCK	2.5×10^{-4}M	0.59	69
		2.5×10^{-5}M	1.20	37
		5.0×10^{-6}M	1.54	19
	TLCK	10×10^{-3}M	0.32	83
		2.5×10^{-3}M	0.76	60
	PMSF	1×10^{-3}M	1.39	27

Cytosol from kidney of adrenalectomized rats was equilibrated with 10^{-9} M ^{3}H-aldosterone (83,000 cpm/ml), Cytosol from HTC cells was equilibrated with 3×10^{-9} M ^{3}H-dexamethasone (98,000 cpm/ml).

TABLE II. Inhibition of Steroid Binding by Protease Inhibitors

Steroid	Inhibitor	Inhibitor concentration	Specifically bound steroid 10^{-13} moles/ml	% Inhibition
Dihydrotestosterone	–	–	0.16	–
	TPCK	2.5×10^{-4}M	0.023	85
		2.5×10^{-5}M	0.088	45
		5.0×10^{-6}M	0.113	29
	TLCK	2.5×10^{-3}M	0.057	64
		5.0×10^{-4}M	0.107	33
	PMSF	1.0×10^{-3}M	0.087	45
17 Beta-estradiol	–	–	2.5	–
	TPCK	2.5×10^{-4}M	2.06	18
	TLCK	10×10^{-3}M	1.26	50
		4×10^{-3}M	1.76	30
	PMSF	1×10^{-3}M	0.48	81
		5×10^{-4}M	1.0	60

Cytosol from S115 cells was equilibrated with 10^{-9}M ^{3}H-dihydrotestosterone (135,000 cpm/ml). Rat alpha-fetoprotein was equilibrated with 10^{-9}M ^{3}H-17 beta-estradiol (112,000 cpm/ml).

TABLE III. Inhibition of Steroid Binding by Protease Inhibitors

Steroid	Inhibitor	Inhibitor concentration	Specifically bound steroid 10^{-13} moles/ml	% Inhibition
Progesterone	–	–	2.3	–
	TPCK	2.5×10^{-4}M	0.32	86
		2.5×10^{-5}M	1.22	47
		5.0×10^{-6}M	1.65	28
	TLCK	2.5×10^{-3}M	1.04	55
	PMSF	1.0×10^{-3}M	1.14	50
		5.0×10^{-4}M	1.53	33

Cytosol from chick oviducts was equilibrated with 10^{-9}M ^{3}H-progesterone (96,500 cpm/ml).

TABLE IV. Inhibition of Steroid Binding by Protease Substrates

	% Inhibition by D-TME			% Inhibition by D-TAME	
Steroid	10 mM	8 mM	1 mM	10 mM	5 mM
Aldosterone		82		93	80
Dexamethasone	57	50		97	77
Dihydrotestosterone	87			100	55
17 beta-estradiol			83	76	55
Progesterone		53		69	54

These steroid binding assays were done under the same conditions as the protease inhibitor experiments reported in Tables I–III.

High concentrations of the protease substrates TME and TAME are required to inhibit steroid binding. In one system, estrogen binding to AFP, TME is a more effective inhibitor of steroid binding than TLCK or TPCK.

The observation that 1 mM TME inhibited E_2 binding to AFP permitted us to test whether the binding site in AFP recognizes the ester structure or the hydrophobic properties of tryptophan. We found that 0.5 mM TME inhibited E_2 binding to AFP by about 70% but that 0.5 mM tryptophan or trytophanamide reduced E_2 binding to AFP by less than 5%. This suggests that a site exists on rat AFP that both recognizes the ester structure and regulates E_2 binding.

We considered the possibility that these compounds might inhibit steroid binding by denaturing the steroid receptor, since it is known that TPCK, TLCK, and PMSF can react with thiol groups in enzymes. However, we did our steroid-binding experiments in the presence of 7 mM 2-mercaptoethanol, a reducing agent. Therefore, it is unlikely that the protease inhibitors or substrates eliminate steroid binding by reacting with thiol groups on the receptor.

In addition, more detailed studies with the progesterone receptor in chick oviduct support the postulate that protease substrates bind to a site on the steroid receptor that regulates steroid binding and that this site is contained in the fragment of about 20,000 molecular weight which also contains the steroid binding site. First, we found that the inhibition of progesterone binding to chick oviduct cytosol by 10 mM TME or 10 mM TAME is reversible. This was accomplished with chick oviduct cytosol that had been incubated with 10^{-9}M progesterone and 10 mM TME or 10 mM TAME to eliminate specific binding. This inhibited cytosol was filtered through a Sephadex G-25 column to remove the unbound TME or TAME. This filtrate could now bind ^{3}H-progesterone (Table V). These results are in agreement with our previous report that inhibition of deoxycorticosterone binding to MDCK cell cytosol by TME or TAME was reversible [1].

TABLE V. Reversibility of Inhibition of Progesterone Binding

	% Inhibition by	
	10 mM D-TME	10 mM D-TAME
a. Inhibitors present with ^{3}H-progesterone-receptor complex for 3 h	51	63
b. Inhibitors in (a) removed by filtration on G-25 Sephadex; followed by re-equilibration with ^{3}H-progesterone for 2 h	11	0

a. Chick oviduct cytosol was incubated with 10^{-9} M ^{3}H-progesterone with or without 10 mM TME, 10 mM TAME, or 3×10^{-7}M progesterone for 3 h. Bound steroid was separated from unbound steroid on Sephadex G-25 columns.

b. 3 ml aliquots of cytosol that had been incubated with 10^{-9}M ^{3}H-progesterone with or without 10 mM TME or 10 mM TAME in (a) were filtered on Sephadex G-25 columns to remove unbound ^{3}H-progesterone, TME, or TAME. Then the filtrate was incubated with 2×10^{-9}M ^{3}H-progesterone with or without 5×10^{-7}M progesterone for 2 h. Bound steroid was separated from unbound steroid on Sephadex G-25 columns.

% inhibition refers to the inhibition of specifically bound counts.

Sherman and co-workers [8–10] have shown that, in the presence of 20 to 100 mM calcium, the progesterone receptor in chick oviduct cytosol is cleaved by protease(s) to form a 20,000 molecular weight mero-receptor [8] that can bind progesterone with the same affinity as the high molecular weight progesterone receptors. We incubated chick oviduct cytosol overnight in 20 mM calcium chloride at 4°, conditions known to result in complete conversion of the progesterone receptor to the 20,000 molecular weight form [8–11]. Complete formation to the mero-receptor was varified by filtration on agarose, as described by Sherman et al [8]. Progesterone binding to this mero-receptor was inhibited by protease inhibitors and substrates to approximately the same extent as shown in Tables III and IV for the native progesterone receptor. This indicates that the locus on the progesterone receptor that recognizes protease inhibitors and substrates is close to and could be identical to the steroid binding site.

Our studies are relevant to work on growth and differentiation in at least two ways. First, studies on the regulation of cell growth by proteases often involve the use of protease inhibitors and substrates to block the activity of cell-associated proteases [12, 13]. It may be erroneous to ascribe the effects of these inhibitors solely to the inhibition of proteolytic activity, for the effects could be due, in part, to reaction with steroid receptors (in particular, the glucocorticoid receptor) that participate in the regulation of cell growth [14–16]. Second, steroid hormones are important regulators of differentiated functions. For example, glucocorticoids induce tyrosine amino transferase in liver cells. We suggest that protease inhibitors and substrates may provide an interesting tool for studying the effects of steroid hormones on development and differentiation.

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