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BINDING AND METABOLISM OF TESTOSTERONE AND OF 5α-DIHYDROTESTOSTERONE IN BULBOCAVERNOSUS/LEVATOR ANI (BCLA) OF MALE RATS: *IN VIVO* AND *IN VITRO* STUDIES

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SUMMARY

1. The binding of labelled compounds after the i.v. administration of tritiated testosterone or of 5α-dihydrotestosterone (5α-DHT) has been demonstrated by agargel electrophoresis at low temperature in the 100,000 *g* cytosol of the rat bulbocavernosus/levator ani muscle complex (BCLA). No binding was found in skeletal muscle (SM). This compared well to a significantly higher uptake of radioactivity by the 100,000 *g* cytosol of the rat bulbocavernosus/levator ani muscle complex (BCLA). No binding was BCLA and SM testosterone itself was the predominant compound (73–77%), the amounts of 5α-DHT (5–7%) being negligible, while in prostate (PR) and seminal vesicles (SV) more than 65% of the testosterone had been converted to 5α-DHT.

2. By *in vitro* studies the characterization of the BCLA cytosol receptor was accomplished as follows: (a) in bulbocavernosus (BC) and levator ani (LA) an identical androgen receptor was found; (b) the receptor showed a higher affinity to 5α-DHT than to testosterone; (c) a plasma contamination was excluded; (d) an inhibition of the specific binding of 5α-DHT and of testosterone was demonstrated by the respective unlabelled compounds and by cyproterone acetate; (e) no displacement was observed with cortisol; (f) under identical conditions no binding occurred in the SM.

3. Binding, uptake and metabolism of testosterone and of 5α-DHT have been compared between PR, SV, BCLA and SM. From the results obtained one may conclude that the androgenic endpoint PR differs from the myotrophic endpoint SM by higher 5α-reductase activities and by larger amounts of specific androgen receptors. Physico-chemical differences between the various receptor proteins could not be found.

INTRODUCTION

Since 1941, when growth stimulation of the perineal muscle group of the male rat was first investigated [1]. the levator ani muscle (LA) as part of this complex has played an important role as a model for testing myotrophic effects of sex hormones. By relating the androgenic potency to the weight increase in prostate (PR) or in seminal vesicles (SV) [2, 3] the quotient of weight increase of LA/weight increase of PR or SV serves as a parameter of the relative activity of a given androgenic-anabolic steroid. Although the validity of the test has been questioned by various authors [4–6] the interest in the LA as a biological model for testing myotrophic or anabolic effects remained. More recently it has been postulated [7] that the LA has two active sites, one for anabolic and one for androgenic activity.

Extensive research on the incorporation of labelled amino acids and of carbohydrates after androgen stimulation of the LA has also been performed [8–12]. On the other side the literature concerning metabolism and binding of androgens in this androgen dependent muscle group is surprisingly scanty. Bruchovsky and Wilson [13] and Gloyna and Wilson [14] reported briefly that after *in vitro* and *in vivo* application of testosterone only trace amounts of 5α-DHT could be detected in the LA. Jung and Baulieu [15] characterized an androgen receptor in LA cytosol *in vitro* which possessed a greater affinity for testosterone than for 5α-DHT.

Our data on both *in vivo* and *in vitro* binding and metablism of testosterone and 5α-DHT in the perineal muscle group are reported in the present paper. The results obtained are compared with the respective findings from PR, SV and skeletal muscle (SM) in order (1) to locate in the androgenic hierarchy the muscle group in question and (2) to discuss androgenic and anabolic potencies of sexual steroids in a broader context.

* Part of doctoral Thesis.

MATERIALS AND METHODS

Chemicals

[1,2-^{3}H]-testosterone (S.A. 45 Ci/mmol, purity 99%), [1,2,6,7-^{3}H]-testosterone (S.A. 91 Ci/mmol, purity 99%) and [1,2-^{3}H]-5α-DHT (S.A. 44 Ci/mmol, purity 98%) were obtained from the New England Nuclear Comp. (NEN). The radioactive solutions were evaporated to dryness and the hormone redissolved in 10% ethanol in 0·9% NaCl solution. Reference substances and unlabelled compounds for competition studies used were as follows: testosterone, 5α-DHT, cortisol, 5α-androstane-3,17-dione, androsterone and epiandrosterone were obtained from Merck, AG, Darmstadt. 5α-androstane-3α,17β-diol (3α-diol), 5α-androstane-3β,17β-diol (3β-diol), 5α-DHT acetate, androsterone acetate and epiandrosterone acetate were obtained from Steraloids. 6-chloro-17-hydroxy-1α,2α-methylene-4,6-pregnadiene-3,20-dione acetate (cyproterone acetate = CYAC) was a gift from Schering, AG, Berlin.

Animals. Male Wistar rats weighing 280–300 g were castrated under ether anaesthesia 40 h before starting the experiments. The organs from at least five animals were pooled for each experiment.

In vivo *studies.* Thirty min before decapitation the castrated rats were injected i.v. into the tail vein with either 250 μCi (approx. 770 ng) [1,2,6,7-^{3}H]-testosterone or 125 μCi (approx. 770 ng) [1,2-^{3}H]-5α-DHT in 1 ml of 0·9% NaCl solution containing 10% (v/v) ethanol. PR, SV and part of the quadriceps femoris muscle (SM) were removed, weighed and immediately placed in an ice bath or were cooled in liquid nitrogen. The BCLA was removed according to Eisenberg and Gordan [2]. Blood was heparinised and centrifuged at 3000 rev./min for 15 min to obtain the plasma.

Thin-layer chromatography. After pooling the respective organs, buffer (0·25 M saccharose, 0·15 M NaH_2PO_4, 0·15 M KH_2PO_4, pH 7·4) was added to the tissue 1:10 (w/v). Homogenization was performed in an Ultra Turrax (type TP 18/2, Fa. Janke u. Kunkel, Staufen, West-Germany) homogenizer in 5 s intervals under continuous cooling in an ice bath. Aliquots of the homogenates and plasma were taken for measuring the total radioactivity, and that remaining was then extracted and separated by t.l.c. on silica gel in a chloroform–acetone (90:10, v/v) system. The androstanediols were further separated by t.l.c. on Al_2O_3 G (type E) in a benzene ethanol (97:3, v/v) system, according to Morfin *et al.* [16]. To separate 5α-DHT from androsterone and epiandrosterone the respective fraction was acetylated and the acetylated steroids were then chromatographed on Al_2O_3 G (type E) in a cyclohexane–ethylacetate (90:10, v/v) system. Further details have been previously reported [17, 18].

Agargel electrophoresis. Two weight volumes tissue +1 volume buffer were pulverized in a porcelain mortar chilled in liquid nitrogen. The fine powder was transferred to centrifuge tube and allowed to stand at 2–4°C until it had thawed. Ultracentrifugation (49,000 rev./min. 100,000 *g*) was performed to obtain the cytosol. Agargel electrophoresis at low temperature was performed according to Wagner [19] and details have been published previously [20].

In vitro *studies.* Unlabelled cytosol and plasma were obtained as mentioned above. Incubation with labelled compounds was performed for 4 h at 2–4°C, the final androgen concentration amounting to 3.10^{-9} M. Competition studies were carried out as follows: the unlabelled compounds dissolved in 0·9% NaCl containing 10% (v/v) ethanol were added *together* with the labelled compounds to the cytosols.

Measurement of radioactivity. (1) Homogenates were dissolved in Soluene 350 (Packard Comp.) and the total radioactivity was measured in a Packard Tricarb liquid scintillation counter. Quenching was corrected by external standardization. (2) t.l.c. fractions analysed in d.p.m. in glass counting vials after addition of scintillation fluid. (3) To facilitate the elution of the radioactivity [19], the structure of the gel was destroyed by freezing it over night in the counting vials, then scintillation fluid was added. After another 12 h the samples were counted without external standardization, because of a constant AES ratio.

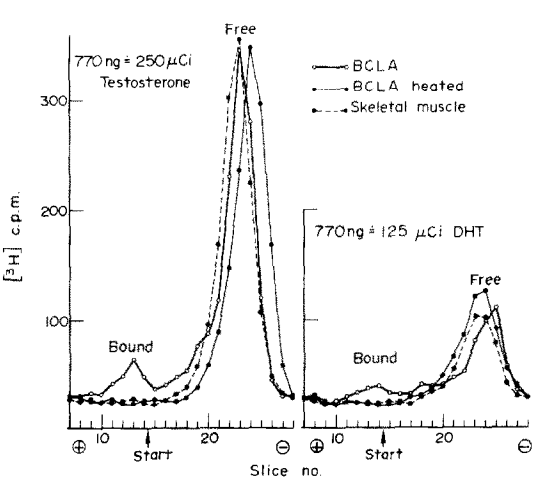

Fig. 1. *In vivo* binding of radioactivity in the 100,000 *g* cytosol of the rat BCLA and SM, analysed by agargel electrophoresis. [^{3}H]-Testosterone or [^{3}H]-5α-dihydrotestosterone were applicated i.v. to each castrated rat 30 min before decapitation. Pooled BCLA and SM of five castrated rats were processed into cytosol. Aliquots were heated at 45°C for 1 h. Forty μl cytosol was applied between slice nos. 14 and 15. Bound hormone migrates to the anode (left), unbound to the cathode (right). Radioactivity *with* background was measured in c.p.m./slice.

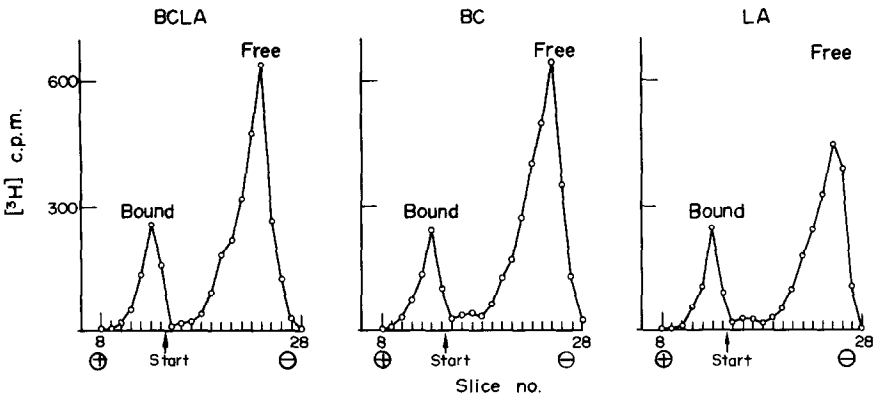

Fig. 2. *In vitro* binding of 5α-dihydrotestosterone in the 100,000 *g* cytosol of the bulbocavernosus/levator ani muscle (BCLA), bulbocavernosus muscle (BC) and levator ani muscle (LA), analysed by agargel electrophoresis. Pooled muscles of five castrated rats were processed into the respective cytosols, which were incubated with $3{\cdot}4 \cdot 10^{-9}$ M 5α-dihydrotesterone for 4 h at 0–2°C. 40 μl cytosol was applied between slice nos. 14 and 15. Bound hormone migrates to the anode (left), unbound to the cathode (right). Radioactivity *without* background was measured in c.p.m./slice.

RESULTS

Binding, uptake and metabolism of i.v. injected testosterone and 5α-DHT in BCLA and SM

Figure 1 summarizes the data of cytosolic bound radioactivity in BCLA and SM after *in vivo* administration of tritiated testosterone or of 5α-DHT. In each of five experiments a heatlabile peak in the anodic part of the agargel electrophoresis was found in BCLA, but not in SM. The very small binding peak after 5α-DHT injection becomes valid only when compared (1) to the left side of Figs. 1 and [2] to our *in vitro* studies (Figs. 2–4). The small peak at slice nos. 18 and 19 after 5α-DHT injection is not constant and often caused by a shifting of radioactivity from the left to the right. The large cathodic peak appearing on the right side from the start represents unbound radioactivity. The uptake of radioactivity (Table 1), both after testosterone and 5α-DHT application was about 40% higher in BC and LA tissue than in SM, while the differences

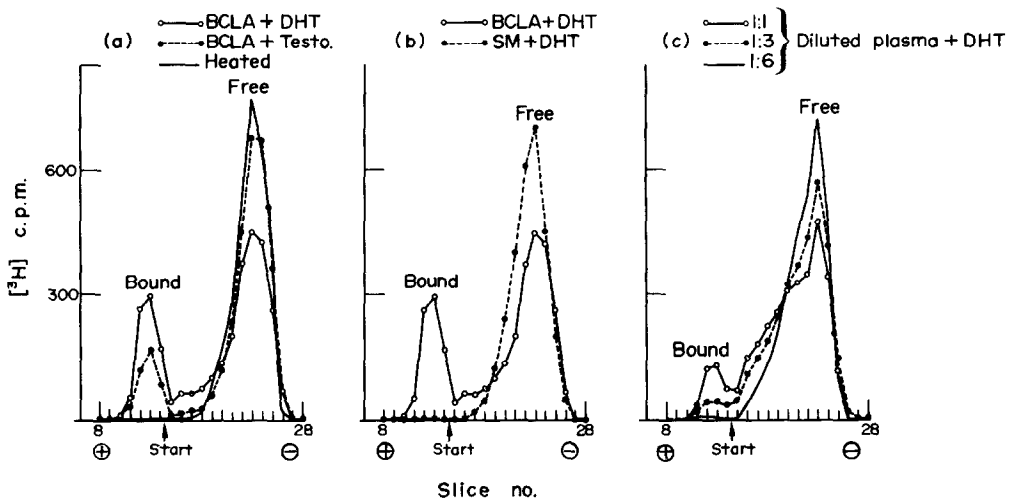

Fig. 3. *In vitro* binding of 5α-dihydrotestosterone and testosterone in the 100,000 *g* cytosol of BCLA (A), compared to SM (B) and plasma (C), analysed by agargel electrophoresis. Pooled muscles and blood of five castrated rats were processed into cytosol and various plasma dilutions respectively, which were incubated with $3{\cdot}4 \cdot 10^{-9}$ M 5α-dihydrotestosterone or $3{\cdot}4 \cdot 10^{-9}$ M testosterone for 4 h at 0–2°C. Aliquots were heated at 45°C for 1 h before incubation starts. 40 μl cytosol was applied between slice nos. 14 and 15. Bound hormone migrates to the anode (left), unbound to the cathode (right). Radioactivity *without* background was measured in c.p.m./slice.

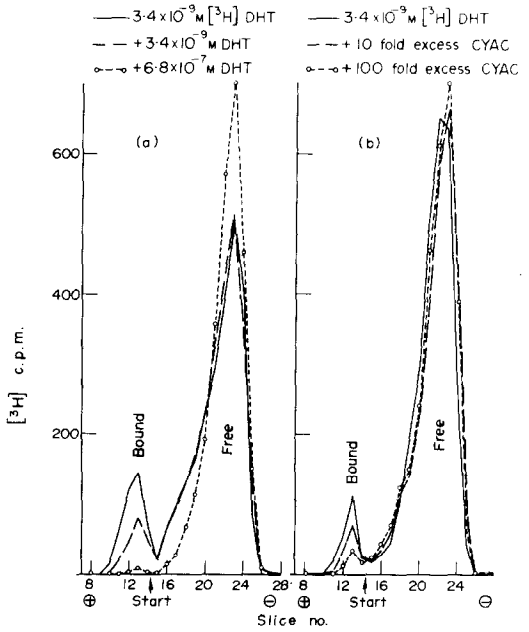

Fig. 4. *In vitro* displacement of the [^{3}H]-5α-dihydrotestosterone binding in BCLA cytosol by cold 5α-dihydrotestosterone (A) and cyproterone acetate (B), analysed by agargel electrophoresis. Pooled BCLA of five castrated rats were processed into 100,000 *g* cytosols (cytosols in A and B were obtained from two different experimental series). Incubation was performed for 4 h at 0 2°C with [^{3}H]-5α-dihydrotestosterone alone or together with the respective cold compounds. Forty μl cytosol was applied between slice nos. 14 and 15. Bound hormone migrates to the anode (left), unbound to the cathode (right). Radioactivity *without* background was measured in c.p.m./slice.

were less marked (13–25%) in the respective cytosol fractions. The percentage distribution of the main metabolites in BC, LA and SM is reported in Table 2. It seems remarkable that after *in vivo* administration of testosterone more than 70% of the steroids extracted from BC, LA and SM is unchanged testosterone, while after 5α-DHT injection a high interconversion to the 3α-diol was demonstrable.

In vitro *characterization of the BCLA receptor*

As shown in Fig. 2 separately processed BC and LA demonstrate an identical electrophoretic pattern of bound and unbound 5α-DHT. The pictures compare well to that shown by the combined-BCLA-cytosol, the following experiments therefore were restricted to BCLA cytosol only. The heatlabile peak of 5α-DHT is greater than that of testosterone (Fig. 3a). In accordance with our *in vivo* studies, in SM no testosterone or 5α-DHT binding was detectable (Fig. 3b). To exclude a plasma contamination as predominant cause for steroid binding, the respective data of various plasma dilutions were directly compared to those of the cytosol (Fig. 3c). Two points should be mentioned: (1) The anodic peak occurring in plasma (Fig. 3c) was always smaller than that in the cytosol (Fig. 3a) and (2) it was influenced neither by heating nor by cyproterone acetate.

Further characterization of the cytosol receptor was accomplished by displacement studies. With equimolar concentration of cold 5α-DHT binding of the labelled substance was inhibited to about 50%. In 200-fold excess the cold compound induced a nearly complete displacement. Cyproterone acetate also competed with 5α-DHT for the receptor, the effect being dose dependent. Cortisol even in a 100-fold excess did not result in any displacement effects. Due to the smaller affinity of testosterone, the characterization of the testosterone receptor did not lead to such clear pictures as demonstrated for the 5α-DHT, the data being in principle, however, comparable.

Growth stimulation of the LA

As may be seen from Table 3 castration leads to a significant decrease of the LA wet weight.

Table 1. Radioactivity in the tissue and cytosol of various muscles 30 min after i.v. administration of 250 μCi [1,2,6,7-^{3}H]-testosterone and 125 μCi [1,2-^{3}H]-5α-dihydrotestosterone to castrated rats. Tissue and cytosol of five animals were pooled. Mean values of at least two experiments are shown with a deviation from the actually obtained values of $<10\%$ and in the case of SM $<15\%$

	Tissue (d.p.m. $\cdot 10^3$/g wet wt)			Cytosol (c.p.m. $\cdot 10^2$/ml)	
	BC	LA	SM	BCLA	SM
Testosterone	459	446	267	512	450
5α-DHT	131	—	79	204	153

BC = bulbocavernosus muscle, LA = levator ani muscle, SM = skeletal muscle, BCLA = bulbocavernosus/levator ani muscle complex.

Table 2. Distribution in % of androgen metabolites obtained by t.l.c. in tissue of various muscles, 30 min after administration of testosterone and 5α-dihydrotestosterone to castrated rats. Tissue of at least five animals were pooled. Mean values of at least two experiments are shown with deviation from actually obtained values of $<5\%$, valid only for mean values $>10\%$

	Radioactive steroid injected					
	Testosterone (m)			5α-DHT (m)		
Metabolite	BC	LA	SM	BC	LA	SM
3α-diol	10	9	10	41	49	50
5α-DHT	7	6	5	35	31	28
Testosterone	74	77	73	<3	<3	<3

3α-diol = 5α-androstane-3α,17β-diol. For further abbreviations see Table 1.

Table 3. Effect of 5α-dihydrotestosterone on LA muscle weight in castrated male rats

No of rats (body wt 300 g)	Condition	Treatment	Levator ani (wet wt mg)
8	castrated 2 days	—	235 ± 15 S.D.
5	castrated 6 days	—	181 ± 24 S.D.
5	castrated 12 days	—	173 ± 19 S.D.
5	castrated 12 days	5α-DHT for 10 days i.p. (5 mg every second day)	299* ± 18 S.D.

* Significant deviation from the above values ($P < 0.001$).

Administration of 5α-DHT results in a significant weight increase when compared to the values of days 2, 6 and 12 after castration.

Comparison of in vivo *data on binding, uptake and metabolism of labelled testosterone in various androgen dependent organs of male rats*

After i.v. testosterone administration the most impressive binding peak was found in the PR cytosol, whereas the binding peaks in SV and BCLA were substantially smaller (Fig. 5). The findings were underlined by recovery data from the respective cytosols. When compared to the data in SV and BCLA (Table 4) about 60–70% more activity was found in the PR cytosol. Regarding the radioactivity found in whole tissue homogenates of PR and SV (Table 4) the difference was far less marked, being probably explained by the fact that SV cytosol contains about 40% less soluble protein than PR cytosol does (Table 5). Finally from the data, summarized in Table 6, it becomes clear that only in PR and SV testosterone is metabolized whereas no substantial interconversion of this hormone occurs in BCLA or in SM.

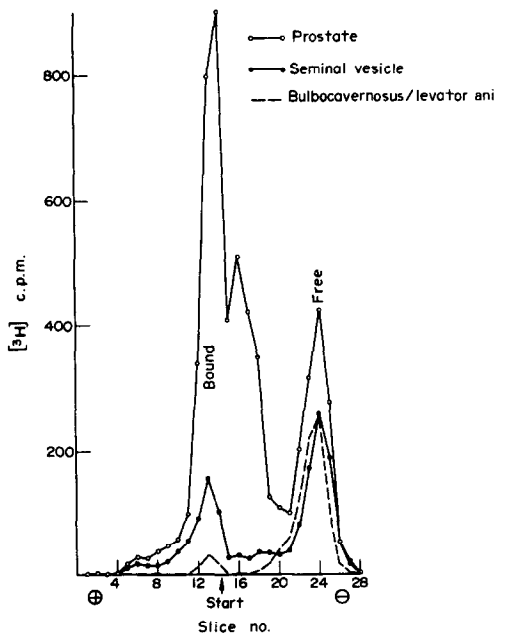

Fig. 5. *In vivo* binding of radioactivity in the 100,000 *g* cytosol of the rat prostate, seminal vesicles and BCLA 30 min after i.v. injection of 250 μCi [1,2,6,7-^{3}H]- (approx. 770 ng) testosterone, analysed by agargel electrophoresis. The pooled organs of five castrated rats were processed into cytosol. Forty μl was applied between slice nos. 14 and 15. Bound hormone migrates to the anode (left), unbound to the cathode (right). Radioactivity *without* background was measured in c.p.m./slice.

DISCUSSION

The discovery of heatlabile bound radioactivity in the BCLA cytosol (Fig. 1) after i.v. application of testosterone and 5α-DHT, and the absence of this binding in the SM correlates well with the higher uptake of radioactivity in BCLA compared to SM (Table 1). Different quantities of receptor protein could be at least partly responsible for the accumulation differences. The question remains open, however, whether SM contains only small amounts of receptor protein, unmeasurable by the available methods, or whether no specific binders are present. Table 1 shows furthermore that in all investigated muscles more radioactivity was recovered after application of testosterone than after 5α-DHT. In SM this is in accordance with previous findings [17]. The question arises if this is due to a greater affinity of BCLA receptor for testosterone than for 5α-DHT. The data demonstrated in Fig. 1, however, allow us, together with our metabolic studies (Table 3), to conclude that more radioactivity must be *bound* in the BCLA after testosterone than after 5α-DHT application. In BCLA due to the lack of 5α-reductase activity [13, 14], in contrast to the PR cytosol [13, 17, 21], after testosterone application 73–77% testosterone and 5–6% 5α-DHT as bindable metabolites were found, while after 5α-DHT injection only about 30% 5α-DHT and trace amounts of testosterone were present. This conclusion is valid only if any

Table 4. Radioactivity in the tissue and cytosol of various organs 30 min after i.v. administration of 250 μCi testosterone to castrated rats. For further details see Table 1

Tissue (d.p.m. $\cdot 10^3$/g wet wt)					Cytosol (c.p.m. $\cdot 10^2$ ml)			
PR	SV	BC	LA	SM	PR	SV	BCLA	SM
1352	1050	459	446	267	1638	700	512	450

PR = prostate, SV = seminal vesicles. For further abbreviations see Table 1.

Table 5. Protein content and radioactivity* per mg protein of the cytosol of various organs of castrated rats

	mg Protein (per ml cytosol)	Radioactivity (per mg protein)
PR (n = 17)	41 ± 5 S.D.	4000 c.p.m.
SV (n = 14)	25 ± 4 S.D.	3500 c.p.m.
BCLA (n = 11)	40 ± 3 S.D.	1300 c.p.m.
SM (n = 16)	37 ± 5 S.D.	1200 c.p.m.

* Radioactivity was calculated by relating the values of Table 4 to protein content. For abbreviations see Tables 1 and 4.

binding of 3α-diol to the receptor in BCLA cytosol is absent (Krieg *et al.*, in preparation), a finding which compares well to the absence of 3α-diol binding in the PR cytosol [22]. Additionally in BCLA cytosol after testosterone application a greater quantity of free radioactivity compared to 5α-DHT injection is present (Fig. 1). One might speculate that in the cell compartments, apart from the specific binding process, membrane processes [22, 23] could be responsible for different ratios of free to bound steroids.

Further characterization of the receptor was achieved by *in vitro* studies. When beginning the series in the separately processed BC and LA cytosol identical binding peaks were obtained. This is in agreement with comparative anatomical and histological experiments [6, 24], and confirms that BC and LA are a single entity. This allows us to speak generally of BCLA, which Wainman and Shipounoff (1) named "perineal muscle group" of the male rat (including the ischiocavernosus muscle). Under identical experimental conditions always more 5α-DHT than testosterone was bound (Fig. 3), which points to a higher affinity of 5α-DHT for the binding protein. The affinity to the receptor therefore does not differ qualitatively from that of the PR [20, 25, 26]. Additionally both the BCLA- (Figs. 3b, 3c and 4) and PR-cytosol receptor [20] are thermolabile, migrate to the same distance in the electrophoresis and are displaced by cyproterone acetate and 5α-DHT. Conflicing data were shown by Jung and Baulieu [15]* who obtained a greater affinity to the LA cytosol receptor with testosterone than with 5α-DHT. Methodological differences in processing and analysis do not allow a direct comparison with our data.

Table 6. Distribution of percentage of androgen metabolites obtained by t.l.c. in tissue of various organs 30 min after administration of testosterone to castrated rats. For further details see Table 2

Metabolite	Organ PR	SV	BC	LA	SM
3α-diol	6	8	10	9	10
5α-DHT	70	65	7	6	5
Testosterone	8	15	74	77	73

For abbreviations see Tables 1 and 4.

Summarizing, the following conclusions are drawn: (1) A specific androgen receptor with greater affinity to 5α-DHT than testosterone exists in the BCLA. Its physico-chemical characteristics correspond well to those of the cytosol receptor in the PR. (2) In BCLA and SM cytosol, practically no 5α-reductase activity was found. This implies that in these muscles testosterone itself must be the active hormone, and this agrees with the data of Buresova and Gutman [10, 11], who in extensive *in vitro* studies proved that testosterone alone can stimulate cellular processes in the LA. (3) If 5α-DHT is administered in comparatively high doses, it will be bound to the receptor and will induce biological activities in the BCLA complex.

Under *in vivo* conditions (Fig. 5, Tables 4–6) a

* Shortly after finishing this paper Mainwaring and Mangan (*J. endocr.* **59** (1973) 121–129) demonstrated under *in vitro* conditions by density gradient ultracentrifugation a specific 5α-DHT receptor in the LA of the guinea-pig but not in the rat.

further point was clarified by a direct comparison between PR and SV on the one side and BCLA and SM on the other: it is not only the greater amount of 5α-DHT in the PR and SV and the higher affinity of the receptor protein to this compound, which may explain uptake and binding differences, but the BCLA cytosol (and speculatively SM) must have substantially less receptor protein than the PR cytosol. It should be mentioned additionally that the uptake and binding differences between PR and SV (Fig. 5, Table 4) will be distorted due to lower protein content of the SV (Table 5). After relating the radioactivity to equal protein concentrations, the uptake differences obtained between PR and SV support findings by other authors [13, 27, 28].

Finally one comes to the conclusion that between the androgenic endpoint PR and myotrophic endpoint SM a quantitative decline of 5α-reductase activity and cytosolic receptor protein exists. From the above SV and BCLA can be located in this androgen dependent hierarchy. It should be mentioned in this context that other enzymes also have variable activities in androgen dependent organs, e.g. 3α,17β-oxidoreductase [29]. Although the question whether in SM an androgen receptor exists at all cannot be answered at the moment, the following speculation may be made: "androgens", which are able in low concentration to saturate the receptor protein in the respective organs, will develop predominantly myotrophic activity, whereas those which through enzyme action are converted into highly active androgen forms, e.g. 5α-DHT and which will then be bound in sufficient quantity to the androgen receptor protein will develop predominantly androgenic activity.

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