

Androgen-Binding Levels in a Sexually Dimorphic Muscle of *Xenopus laevis*¹

NEIL SEGIL, LAWRENCE SILVERMAN,² AND DARCY B. KELLEY³

Department of Biological Sciences, Sherman Fairchild Building, Columbia University,
New York, New York 10027

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The larynx of adult South African clawed frogs, *Xenopus laevis*, is larger in males than in females and hypertrophies in adult females and juveniles in response to androgen. Sexual dimorphism and androgen sensitivity suggest that the larynx is a testosterone target tissue. Saturation analysis of androgen (R1881) binding in laryngeal cytosol revealed an approximately threefold quantitative difference between male and female androgen-binding levels (36.4 vs 11.5 fm/mg protein). By contrast, as measured by one-point assay, androgen-binding levels in thigh muscle of either males or females were between 0 and 4 fm/mg protein with no apparent sex difference. Competition studies indicated that dihydrotestosterone was the most effective competitor for R1881 binding activity in the larynx. Saturation analysis showed the binding activity to be saturable and of high affinity (apparent K_d 0.46 nM in the male and 0.38 nM in the female). After 1 month of testosterone treatment, female binding levels averaged 16.6 fm/mg protein with a K_d of 0.49 nM, within the range for normal females. In males castrated for 4 months, binding levels were 52 fm/mg protein. After 1 year of castration, binding levels were 25 fm/mg protein. We conclude that laryngeal muscle is an androgen target tissue with sexually dimorphic levels of binding in adults. © 1987 Academic Press, Inc.

Sex differences in the morphology and overall mass of vertebrate skeletal muscle are thought to be brought about by the action of gonadal steroids, in particular the androgens (Papanicolaou and Falk, 1938). Development and maintenance of these differences are dependent on the presence of a functional androgen-specific receptor molecule (Gustafsson *et al.*, 1983; Max, 1981; Breedlove and Arnold, 1981). In some animals, muscles used in sexual behavior have been shown to be specialized androgen target tissues with levels of androgen receptor well above those of other

skeletal muscle. The syrinx of song birds (Lieberburg and Nottebohm, 1979) and the levator ani/bulbocavernosus of rodents (Jung and Baulieu, 1972) are examples. The larynx of *Xenopus laevis* produces sex-typical vocalizations (Ridewood, 1898; Tobias and Kelley, 1985). Laryngeal muscles of the South African clawed frog are three times larger in the adult male than in the female (Sassoon and Kelley, 1986; Ride-wood, 1898). Adult female laryngeal muscle undergoes hypertrophy in response to exogenously administered testosterone (Sassoon and Kelley, 1986). Sexually immature laryngeal muscle in both sexes responds to administered testosterone by enlargement through stimulated myoblast proliferation and hypertrophy (Sassoon *et al.*, 1986).

To determine which factors underlie laryngeal muscle sensitivity to testosterone, we compare adult male and female androgen binding in the cytosolic fraction taken from male and female laryngeal

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² Present address: UMDNJ-Rutgers Medical School, Piscataway, NJ 08854.

³ To whom reprint requests should be addressed, at Department of Biological Sciences, 1018 Sherman Fairchild Building, Columbia University, New York, NY 10027.

muscle as well as determine binding levels in thigh muscle of both sexes. Toward the same end, we study the effect of testosterone treatment on androgen-binding levels in females and castration on binding levels in males.

MATERIALS AND METHODS

Several groups of animals were compared in this study: adult males, adult females, androgen-treated females, and castrated males. Each assay used pooled tissue from two to eight animals depending on how much cytosol was needed for the particular experiment. Androgen-binding activity was assayed by saturation analysis to assess maximum binding capacity (B_{max}) and apparent dissociation constants (K_d 's). The specificity of [3 H]R1881 binding to cytosolic macromolecules was assayed by a set of competition experiments performed with testosterone (T), dihydrotestosterone (DHT), estradiol (E), progesterone (P), corticosterone (C), and triamcinolone acetonide (TA) (Sigma Chemical).

Animals and hormone treatment. Adult *X. laevis* were purchased from Xenopus I (Ann Arbor, MI). Males weighed on average 37 ± 1.6 g (\pm standard deviation), and females 80 ± 8.0 g. Receptor assays were performed on sexually mature males gonadectomized 1 week prior to assay to reduce endogenous steroid levels which would otherwise compete in binding studies. Receptor assays were also performed on sexually mature females and on females that had been treated with testosterone. Adult females were ovariectomized 1 week prior to assay. Testosterone-treated females were ovariectomized and a testosterone pellet (0.25 mg/g body wt) was implanted into the dorsal lymph sac. Testosterone-treated females retained the pellet implant for 1 month; greater than 99% of each pellet was recovered when the pellet was removed. One week prior to assay, these pellets were removed to clear circulating steroid which might otherwise interfere with the binding assay. Two groups of males that had undergone castration were assayed, one approximately 1 year after castration and another group 4 months after castration.

Cytosol preparation. All procedures were carried out at 4°. Animals were anesthetized by hypothermia and decapitated, and then larynges and thigh muscle (gluteus) were removed and dissected free of fat and adherent connective tissue. Larynges were then weighed and the bipinnate muscle was stripped from laryngeal cartilage. Muscle was minced and then homogenized in TEGMD buffer (pH 7.4, 0.01 M Tris, 1.5 mM EDTA, 10% glycerol, 0.02 M sodium molybdate, 1.0 mM dithiothreitol; Sigma). The homogenate was centrifuged at 40,000 rpm (140,000g) for 45 min in an SW 50.1 rotor (Beckman) and the supernatant (minus

lipid) was removed for preparation of incubates. The protein concentration of the cytosol was adjusted to between 0.5 and 1.0 mg/ml and absolute values were later determined according to the method of Bradford (1976).

Saturation analysis. Laryngeal muscle cytosol preparations were incubated with radioactive methyltrienolone, [3 H]R1881 (87 Ci/mmol, New England Nuclear), an androgen analog with reported high affinity for the androgen receptor (Hicks and Walsh, 1979) which does not bind to serum proteins in rodents (Bonne and Raynaud, 1975). Concentrations ranging from 0.1 to 10 nM were used and parallel incubates containing a 100-fold excess of nonradioactive R1881 controlled for nonspecific binding. To establish that assays were performed at equilibrium, in one series of experiments incubates were sampled hourly for 0–6 hr. Equilibrium (maximum binding) was reached by 1 hr of incubation and did not change over the next 5 hr (data not shown). Incubations were carried out at 4° for 4 hr. [3 H]R1881 bound to macromolecules was separated from unbound steroid by gel filtration on Sephadex LH-20 (Sigma) minicolumns (bed vol = 0.4 ml) as described by Ginsburg *et al.* (1974), with modifications (Sheets *et al.*, 1985). The columns were eluted with TEGMD buffer and the macromolecular fraction was collected and counted in a Beckman liquid scintillation counter with HistoFluor (National Diagnostics, Somerville, NY). Counter efficiency was determined at the time of each assay with a [3 H]toluene standard (NEN). The apparent maximum binding (B_{max}) and dissociation constant (K_d) were obtained by a standard Scatchard analysis (Scatchard, 1949). Binding activity in thigh was determined by a series of one-point assays. Determinations were carried out as above except that concentrations of approximately 10 nM [3 H]R1881 were used in the incubates with and without a 100-fold excess of unlabeled R1881.

Ligand specificity. To assay for specificity of R1881 binding, a series of competition experiments were run. Assays were conducted as in the one-point assays except that instead of the nonradioactive R1881, competitors in the form of T, DHT, E, P, C, and TA were included in the incubation mix at concentrations in 1-, 10-, and 100-fold excess of [3 H]R1881. [3 H]R1881 concentrations were held constant at approximately 10 nM. In preliminary assays, incubates were run in parallel with and without TA. No differences in binding were observed (data not shown).

RESULTS

Laryngeal Muscle Is an Androgen Target Tissue

Assays were performed to assess the presence and relative levels of androgen

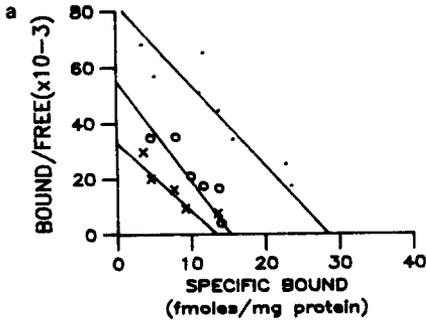


FIG. 1a. Scatchard analysis: Saturation analysis of [³H]R1881 binding in male, female, and testosterone-treated female laryngeal muscle plotted by the method of Scatchard (1949). The male assay used pooled tissue from five animals, while the female assays used pooled tissue from six to eight animals. The K_d and B_{max} are derived from a least-squares analysis of the transformed data. The K_d from the male assay is 0.34 nM, from the female is 0.41 nM, and from the testosterone-treated female is 0.28 nM. The B_{max} from the male is 28.5 fm/mg protein, from the female is 13.4 fm/mg protein, and from the testosterone-treated female is 15.3 fm/mg protein.

binding in the laryngeal bipinnate muscle. Figure 1a is a Scatchard plot of data derived from representative saturation analyses of male, female, and testosterone-treated female laryngeal cytosol. These results indicate that the binding is saturable and of high affinity. The apparent K_d in

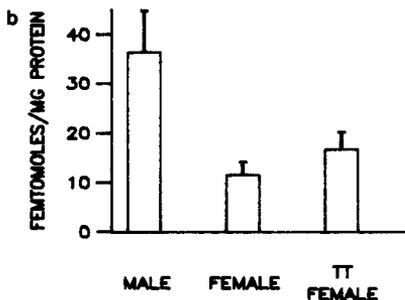


FIG. 1b. Androgen-binding activity in laryngeal muscle from males, females, and testosterone-treated females. The B_{max} values from three separate assays on male animals were averaged to establish laryngeal muscle binding levels (35.4 ± 8.3 fm/mg protein). Two separate assays were averaged for the females (11.5 ± 2.6 fm/mg protein) and three separate assays for the testosterone-treated females (16.6 ± 3.5 fm/mg protein).



FIG. 1c. Androgen-binding activity in thigh muscle from males, females, and testosterone-treated females. Androgen-binding activity in the thigh was established by one-point assays. Several concentrations of [³H]R1881 above saturation were used to assay the total and nonspecific binding activity. Average values from the saturated incubates are shown: males, 2.17 ± 0.85 ; females, 3.7 ± 3.0 ; and testosterone-treated females, 2.2 ± 1.8 fm/mg protein.

these assays was 0.34 nM for the male, 0.41 nM for the female, and 0.28 nM for the testosterone-treated female. The average K_d for males was 0.46 nM ($N = 3$ assays); for females it was 0.38 nM ($N = 2$) and for testosterone-treated females 0.49 nM ($N = 3$). Figure 1b illustrates the cumulative results of all assays. Males had a mean B_{max} of 36.4 ± 8.3 fm/mg protein, females 11.5 ± 2.5 fm/mg protein, and testosterone-treated females 16.7 ± 3.5 fm/mg protein. The results of assays on two groups of males castrated for either 4 or 12 months differed. After 4 months of castration, animals had binding levels of 52 fm/mg protein while the B_{max} from the assay of the 12-month castrates was 25 fm/mg protein.

Figure 1c illustrates the results of one-point assays on thigh muscle from male, female and testosterone-treated females. Males averaged 2.17 ± 0.85 fm/mg protein ($N = 4$ assays), females averaged 3.7 ± 3.0 fm/mg protein ($N = 3$), and testosterone-treated females averaged 2.2 ± 1.8 fm/mg protein ($N = 2$). In several instances, androgen binding in thigh was undetectable by our assay.

Figure 2 illustrates the specificity of R1881 binding in male laryngeal muscle cy-

tosol. At 10 times the concentration of radiolabeled R1881, nonradioactive R1881 and DHT compete equally well (76 and 78% of total, respectively). At this concentration T, E, and P all compete poorly for [^3H]R1881 binding (28, 28, and 23%, respectively). Competition by corticosterone was 14% and by triamcinolone acetonide was 4%. Competition results for testosterone-treated female laryngeal muscle cytosol were very similar (data not shown). This hierarchy of steroid competition was confirmed for males and testosterone-treated females in an extensive series of one-point assays (data not shown). In females, assays confirmed that DHT was the best competitor; however, the relative effectiveness of the other competitors was more variable.

These results indicate that (1) there is a specific androgen-binding component in the laryngeal muscle of *X. laevis*, (2) per milligram protein there is more androgen binding in male than in female laryngeal muscle, and (3) both male and female laryngeal muscle contain significantly higher

levels of androgen binding than does thigh muscle.

Effects of Endocrine Manipulations on Laryngeal Weights

Male and female laryngeal weights are dimorphic: the average for males is 0.42 ± 0.05 g ($N = 22$) and for females is 0.12 ± 0.02 g ($N = 16$). Following 1 month of testosterone treatment, female laryngeal weight increased to 0.24 ± 0.04 g ($N = 8$). After 5 to 6 months of treatment, female laryngeal weight increased further to 0.32 ± 0.04 g ($N = 9$). The overall weight of the male larynx was unchanged 1 year after gonadectomy (0.38 ± 0.04 g; $N = 7$).

DISCUSSION

Our results indicate that laryngeal tissue of adult gonadectomized *X. laevis* contains a macromolecular component that binds the synthetic androgen R1881 with high affinity (nanomolar K_d 's). Muscle sensitivity

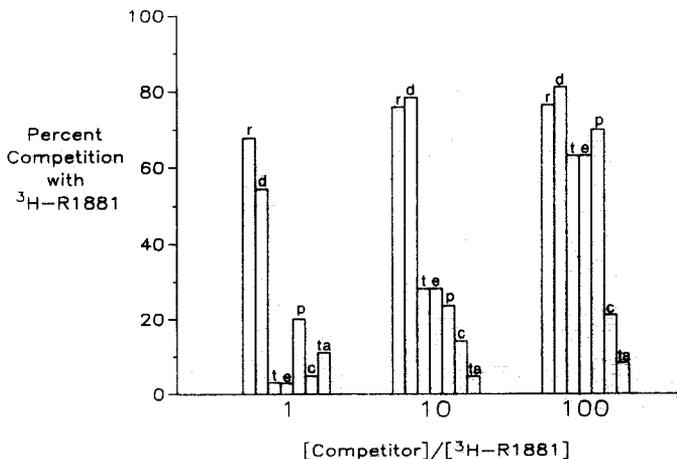


FIG. 2. Specificity of binding activity. Concentration of [^3H]R1881 was held constant at or above saturation (10 nM). Excess of competitors (1-, 10-, and 100-fold) were incubated in duplicate with [^3H]R1881 and cytosol prepared from laryngeal tissue from adult male frogs. Competitors included dihydrotestosterone (d), testosterone (t), estradiol (e), progesterone (p), corticosterone (c), and triamcinolone acetonide (ta).

to circulating androgen is a well-known phenomenon which underlies muscle growth during puberty and atrophy following castration (Pajunen *et al.*, 1982). In *X. laevis*, 1 month of testosterone treatment increased laryngeal weight of gonadectomized females 1.3-fold and 6 months of testosterone treatment resulted in a 1.7-fold increase. These results are comparable to testosterone effects on syringeal muscle weight of adult female zebra finches or canaries which increase by 1.5 and 1.7 times, respectively, in response to 1 month of testosterone treatment (Luine *et al.*, 1980). Note, however, that laryngeal weight of adult male *X. laevis* was not decreased 1 year after gonad removal. Following castration of males, levels of serum androgen drop below detectable levels (Lambdin and Kelley, 1986). Thus the observed sexual dimorphism in laryngeal weight does not require continued high levels of androgen secretion in the adult male.

Jung and Baulieu first reported the existence of a muscle androgen receptor in 1972 and it appears that all or at least most vertebrate skeletal muscles contain androgen receptors (Gustafsson *et al.*, 1984). As in other steroid-responsive cells (Alberts and Yamamoto, 1976), it is assumed that these receptors mediate the changes observed in response to different hormone states although evidence of direct action in skeletal muscle is not yet available.

In several species, sexually dimorphic muscles play an important role in male-specific courtship or copulatory behavior. In these cases, male muscle mass typically exceeds that of females. Examples include the levator ani/bulbocavernosus complex of rats (Cihak *et al.*, 1970), the temporalis muscle of guinea pigs (Kochakian *et al.*, 1964), and the syrinx of songbirds (see above; Luine *et al.*, 1980). In at least two of these examples, levator ani and syrinx, androgen receptor levels are several times higher than those found in other striated

muscle. For instance, Dube *et al.* (1976) reported an approximately threefold difference in DHT binding between thigh (5.3 fm/mg protein) and levator ani (14.5 fm/mg protein). In the syrinx of the zebra finch, 40–50 fm/mg protein of androgen-binding activity was measured in the whole syrinx and approximately 15 fm/mg protein of binding activity was observed in the muscle alone (Lieberburg and Nottebohm, 1979). In that report, no androgen-binding activity was detected in several other muscles which are not sexually dimorphic. It thus appears that, in comparison with other skeletal muscles, sexually dimorphic muscles have an elevated level of androgen binding. Our study indicates that the laryngeal muscle of *X. laevis* belongs to this group of specialized sexually dimorphic skeletal muscles in terms of dimorphism and high levels of androgen binding.

Levels of androgen binding in *X. laevis* laryngeal muscle are sexually dimorphic: values in males are three times those in females. Sex differences in androgen binding have not yet been described in other sexually dimorphic muscle. For example, levels in the syrinx of male and female zebra finches and canaries are not significantly different (Lieberburg and Nottebohm, 1979). The rat levator ani muscle has high levels of androgen receptor in males (Jung and Baulieu, 1972); the muscle is missing in adult females. In nondimorphic skeletal muscle, some studies report lower levels of androgen receptor in males than in females (reviewed in Gustafsson *et al.*, 1984). These measurements, however, were conducted in intact males in which endogenous steroid might be expected to compete for binding sites; castration of males increases apparent androgen binding (e.g., Dahlberg *et al.*, 1981).

Our binding data are consistent with the presence of a high-affinity androgen receptor in laryngeal cytosolic preparations. The binding activity is reduced greatly by

competition with the androgen, dihydrotestosterone, but not by competition with corticosterone or triamcinolone acetonide, a compound that binds to glucocorticoid and progesterone receptors (Zava *et al.*, 1979). These characteristics are similar to those reported for other tissues containing androgen receptor including ventral prostate and skeletal muscle (Wilson and French, 1970; Jung and Baulieu, 1972). However, certain aspects of the competition for binding by unlabeled steroid are puzzling. For instance, T is a much poorer competitor for binding than might be expected for an androgen receptor. We have recently conducted a set of studies on juvenile larynx with radiolabeled DHT instead of R1881 and have obtained similar results in our competition assays (Sassoon *et al.*, 1985). In most mammals, DHT arises principally by the metabolism of T (due to 5α -reductase activity) in peripheral tissues (e.g., Wilson and Walker, 1969). There is little 5α -reductase in muscle (Wilson and Gloyna, 1970; Longcope and Fineberg, 1985), leading to the belief that T is the active form of androgen in this tissue type. In *X. laevis*, however, T and DHT circulate at approximately equal levels in males (Kelley, 1980). Thus circulating DHT could actually be the endogenously active androgen in *X. laevis* laryngeal muscle.

Estradiol also competes for R1881 binding. The level of competition that we observed is also found in better characterized androgen target tissues (for example, see Wilson and French, 1976). No specific estrogen binding was observed when assays were run on laryngeal cytosol preparations from juvenile animals (Sassoon and O'Dougherty, unpublished observation). In addition, circulating estrogen levels in adult males are thought to be extremely low (R. Wallace, personal communication; P. Licht, personal communication) and thus unlikely to interfere with androgen binding.

We have shown previously that little or

no cellular proliferation occurs in adult laryngeal muscle in response to androgen administration (i.e., no new myonuclei) (Sassoon *et al.*, 1986) even though such treatment causes an increase in laryngeal mass in females (this paper). Since the amount of binding/milligram protein stays the same in a female treated with testosterone, the amount of binding/nuclei must increase commensurate with the increase in muscle protein. Thus a steady-state concentration of binding/unit volume of cytoplasm may be necessary for the maintenance of androgen responsiveness.

A recent report from our laboratory suggests that there is a decidedly different cellular response to testosterone administration in the larynx of juvenile (postmetamorphic, sexually immature) frogs as compared with adult animals (Sassoon *et al.*, 1986). In the juvenile frog, testosterone administration rapidly stimulates myogenesis and chondrogenesis. In adults, although the larynx hypertrophies in response to testosterone treatment, no increase in the low level of cell division is observed using autoradiographic methods. Investigation of androgen-binding levels during sexually immature stages of *X. laevis* may reveal correlations between changes in binding levels and the change in cell populations (myogenic precursors to adult muscle) which accompanies this developmental transition.

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