Cytosolic androgen receptor in regenerating rat levator ani muscle

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The development of the cytosolic androgen receptor was studied after degeneration and regeneration of the rat levator ani muscle after a crush lesion. Muscle regeneration appears to recapitulate myogenesis in many respects. It therefore provides a model tissue in sufficiently large quantity for investigating the ontogenesis of the androgen receptor. The receptor in the cytosol of the normal levator ani muscle has binding characteristics similar to those of the cytosolic receptor in other androgen-sensitive tissues. By day 3 after a crush lesion of the levator ani muscle, androgen binding decreased to 25% of control values. This decrease was followed by a 4–5-fold increase in hormone binding, which attained control values by day 7 after crush. Androgen binding remained stable at the control value up to day 60 after crushing. These results were correlated with the morphological development of the regenerating muscle after crushing. It is concluded that there is little, if any, androgen receptor present in the early myoblastic stages of regeneration; rather, synthesis of the receptor may occur after the fusion of myoblasts and during the differentiation of myotubes into cross-striated muscle fibres.

The rat levator ani muscle is remarkably sensitive to androgens. It is present at birth in rats of both sexes. In females, the muscle disappears as they approach puberty but persists if the animals are given androgens. In males, the muscle grows as animals approach puberty but atrophies if the rats are castrated. Conversely, the muscle undergoes hypertrophy if intact male rats are given androgens (Hanzlikova *et al.*, 1970; Gutmann *et al.*, 1967; Cihak *et al.*, 1970). Hormone sensitivity is reflected by the presence of androgen receptors in the cytosol of the levator ani muscle (Jung & Baulieu, 1972; Gustafsson & Pousette, 1975; Dubé *et al.*, 1976; Tremblay *et al.*, 1977; Krieg *et al.*, 1980). There are greater numbers of receptors in the levator ani muscle than in ordinary skeletal muscle (Krieg *et al.*, 1980; Michel & Baulieu, 1980; Krieg, 1976), but fewer than in hormone-sensitive avian syringeal muscles (Lieberburg & Nottebohm, 1979) and in classical androgen target tissues, such as the ventral prostate gland (Schein *et al.*, 1978; Krieg *et al.*, 1980). It should be noted that most investigators

studied the levator ani-bulbocavernosus complex rather than the levator ani muscle alone. Because of its sensitivity to androgens, the levator ani muscle provides a valuable model tissue for studying the effects of sex steroids on muscle. Recently, the levator ani muscle was shown to undergo regeneration after crush injury or after transposition to the bed of the extensor digitorum longus muscle (Gutmann & Carlson, 1978; Carlson *et al.*, 1979). Regeneration of the levator ani muscle was markedly stimulated by androgens (Gutmann & Carlson, 1978). Indeed, an androgen-sensitive muscle, such as the levator ani, appears to be one of the best available donors for muscle transplantation in rats (Gutmann & Carlson, 1978).

To date, there have been no reports on the development of androgen receptors in muscle. An important feature of muscle regeneration is that it appears to recapitulate myogenesis in many respects (Carlson, 1979). In the present study we have taken advantage of this feature and have employed it in the study of the ontogenesis of the cytosolic androgen receptor. The study was designed to answer the following questions. (1) Are androgen receptors present at all stages of regeneration of the levator ani muscle after crush injury? (2) If not, at what stage of myogenesis does the receptor appear?

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Materials and methods

Materials

[17 α -Methyl-³H]methyltrienolone, unlabelled methyltrienolone and liquid-scintillation-counting solution (Aquasol) were purchased from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals

Receptor characterization. Male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) weighing 150–300 g were used. To have sufficient tissue for assay, muscles were pooled. Thus each determination (see below) represents pooling of two to three levator ani muscles. Rats were castrated 4 days before use.

Regeneration study. This portion of the study was conducted on 117 male Sprague–Dawley rats obtained from Charles River Breeding Laboratories. The animals were 49–51 days old at the time of the operation.

Receptor binding

Preparation of cytosol. Muscles were placed in a 10 ml beaker on crushed ice in homogenization buffer (0.05 mM-Tris, 0.1 mM-EDTA, 10% glycerol, 0.25 mM-dithiothreitol, pH 7.4) at concentrations of at least 100 mg (fresh weight) of muscle/ml of homogenization buffer. All subsequent work was carried out on crushed ice in the cold room at 0–4°C.

Muscles were finely minced with scissors and were then homogenized by hand as gently as possible with eight strokes in a Tenbroeck homogenizer (Bellco Glass Co.). The resulting homogenate was centrifuged at 111000 g (r_{av} , 6.52 cm) in a Sorvall OTD-50 preparative ultracentrifuge for 1 h at 2°C. The supernatant fraction, or cytosol, was removed carefully with a Pasteur pipette so as not to include the superficial fatty layer and was used immediately.

Androgen receptor binding assay

[17 α -Me-³H]Methyltrienolone (sp. radioactivity 87 Ci/mmol) was used as the ligand because it binds selectively to the androgen receptor (Bonne & Raynaud, 1976; Tremblay *et al.*, 1977), is not metabolized (Bonne & Raynaud, 1976; Tremblay *et al.*, 1977) and displays no specific binding to serum proteins (Bonne & Raynaud, 1975; S. R. Max, unpublished work). [17 α -Me-³H]Methyltrienolone was added to disposable glass culture tubes (10 mm $\times$ 75 mm) at final concentrations of 0.1–10 nM. A parallel series of tubes contained a 100-fold molar excess of unlabelled methyltrienolone. All tubes also contained a 500-fold molar excess of

triamcinolone acetonide to prevent possible binding of methyltrienolone to a progesterin receptor (Zava *et al.*, 1979). In fact, triamcinolone acetonide caused a 20% displacement of [17 α -Me-³H]methyltrienolone in a competition experiment, indicating the possible existence of a progesterin receptor in the levator ani muscle (S. R. Max, unpublished work). Cytosol (250 μ l) was added to all tubes except blanks, which received 250 μ l of homogenization buffer instead. After gentle vortex-mixing, the tubes were covered and kept in crushed ice in the cold room for 4 h (sufficient time for equilibrium).

Separation of [17 α -Me-³H]methyltrienolone bound to the cytosolic receptor from unbound [17 α -Me-³H]methyltrienolone was accomplished by using two different procedures. In the first procedure, 200 μ l of cystol incubated for 4 h was added to glass tubes (10 mm $\times$ 75 mm) containing a pellet prepared from 800 μ l of a suspension of 0.5% charcoal and 0.05% dextran in homogenization buffer (cf. Clark & Peck, 1979). The pellet had been washed and re-centrifuged several times to remove fine particles. These tubes were incubated for 10 min in the cold, vortex-mixed and centrifuged at 1200 g (r_{av} , 16.41 cm) in a Sorvall GLC-2B centrifuge at 4°C. A portion of the resulting supernatant fraction (100 μ l) was added to 10 ml of Aquasol aqueous liquidfluor, and radioactivity was determined at 30% counting efficiency in a Beckman LS-235 liquid-scintillation spectrometer. Specific binding was determined by subtracting radioactivity in tubes containing a 100-fold molar excess of methyltrienolone (non-specific binding) from that in tubes containing only [17 α -Me-³H]methyltrienolone (total binding). The second method of separating bound from unbound hormones employed Sephadex LH-20, as described by Ginsberg *et al.* (1974). In this method, 200 μ l of cytosol, after incubation with labelled and unlabelled ligands, was added to a 7.0 cm column (Pasteur pipette) of Sephadex LH-20 in homogenization buffer. The cytosol was washed into the column with an additional 100 μ l of homogenization buffer, the column was allowed to stand for 30 min in the cold and 700 μ l of homogenization buffer was then added. The eluted material (void volume) was collected in 20 ml counting vials to which 10 ml of Aquasol was added. Radioactivity was determined as described above. [17 α -Me-³H]Methyltrienolone specific binding was the same with the charcoal–dextran and Sephadex LH-20 assays.

Competition studies were carried out by including competing ligands in tubes containing [17 α -Me-³H]methyltrienolone at 10 nM (i.e. saturating concentration; see below) and comparing specific binding in the presence of competitor with that in its absence (see below). For studies of androgen receptor binding in the levator ani muscle undergo-

ing regeneration, we used a 'one-point' assay, i.e. binding of 10 nM-[17 α -Me-³H]methyltrienolone in the presence and absence of a 100-fold molar excess of unlabelled methyltrienolone (and in the presence of a 500-fold molar excess of triamcinolone acetonide). The use of a 'one-point' assay was necessary because the small amount of muscle tissue available precluded determination of a binding isotherm at each stage of regeneration.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Surgical procedures

The rats were anaesthetized with diethyl ether, and the levator ani muscle was exposed through a midline slit in the scrotum. After being defined by blunt dissection, the muscle was crushed by means of overlapping pinches with a haemostat, as described previously (Gutmann & Carlson, 1978). Care was taken not to injure the motor branches of the pudendal nerve before they entered the muscle. The crushing procedure causes the degeneration and subsequent regeneration of the muscle fibres (Gutmann & Carlson, 1978). At 2, 3, 7, 14, 30 and 60 days after crushing, the levator ani muscles were removed, weighed and immediately frozen in a mixture of solid CO₂ and isopentane. A parallel series of muscles was removed from control animals. The frozen muscles were placed in vials on dry ice and shipped from Ann Arbor to Baltimore via air express. Receptor binding was found to be stable in muscles frozen at -70°C for at least 4 days.

All rats were castrated before removal of the levator ani muscles to remove endogenous androgen bound to cytosolic receptors. For the 2- and 3-day groups, castration was performed 2 days before removal of the muscles. In the other groups (7, 14, 30 and 60 days), castration was performed 4 days before muscle removal.

Histological study

At least one muscle from each group was prepared for histological study. Pieces of levator ani muscles were fixed in Bouin's solution, embedded in paraffin, sectioned at 7 μ m and stained with Ehrlich's haematoxylin and eosin stain or Palmgren's silver stain.

Statistical analysis

Hormone binding of experimental and control muscles was compared by factorial analysis of variance for weighted means, and Dunnett's *t* test (Winer, 1962).

Results

Most studies of androgen receptor binding in the levator ani muscle have employed both the levator ani and the bulbocavernosus muscles together. The levator ani is a 'white' muscle composed histologically of a homogeneous population of fast glycolytic fibres; the bulbocavernosus is a 'red' muscle composed of a mixture of fast glycolytic and fast-oxidative glycolytic fibres (S. R. Max, unpublished work; Hanzlikova *et al.*, 1970). Because only the levator ani muscle was used in the present study, it was necessary to characterize androgen binding to the levator ani alone. Fig. 1 is a binding isotherm for [17 α -Me-³H]methyltrienolone to the cytosol fraction of the levator ani muscle from 4-day castrated adult male rats. The Figure shows [17 α -Me-³H]methyltrienolone specific binding to the levator ani muscle to be saturable, as expected for a physiologically relevant receptor. The data from which Fig. 1 was derived were subjected to analysis by the method of Scatchard (1949) (Fig. 2). This analysis resulted in a straight line, indicating that the levator ani muscle contains a single class of cytosolic androgen receptor molecules. The data of Fig. 2 yield an apparent K_D of 0.74 nM and a maximum binding (B_{max}) of 8 fmol/mg of protein, showing the receptor to be of low capacity and high affinity. As expected for an androgen receptor, this binding component is heat-labile; incubation of cytosol at 37°C for 30 min before adding it to the ligand completely eliminated specific methyltrienolone binding (S. R. Max, unpublished work). Furthermore, binding was linear within the ranges of protein

Fig. 1. Isotherm for [³H]methyltrienolone specific binding to cytosol of rat levator ani muscle. Experimental procedures are as described in the text.

Fig. 2. Scatchard analysis of binding data from which Fig. 1 was derived
 $r^2 = 0.92$.

Fig. 3. Competition by cyproterone acetate for specific binding of $[^3\text{H}]$ methyltrienolone to the rat levator ani muscle cytosolic receptor

Experimental procedures are described in the text. $[17\alpha\text{-Me-}^3\text{H}]$ Methyltrienolone (10nM) was used in all tubes.

concentration used in the present study (S. R. Max, unpublished work).

Further indication that we are measuring a selective cytosolic androgen receptor is given by the blockade of methyltrienolone binding by cyproterone acetate, a well-known androgen receptor antagonist (Fig. 3), and by the data of Table 1. These data show competition for binding of $[17\alpha\text{-Me-}^3\text{H}]$ methyltrienolone by testosterone and

Table 1. Competition by steroid hormones for binding the cytosolic androgen receptor of rat levator ani muscle to $[^3\text{H}]$ methyltrienolone

Experimental procedures are described in the text.

Competitor	Competitor concn. (μM)	Specific binding (%)	
		1	10
Methyltrienolone*		0	0
Testosterone		20	16
5 α -Dihydrotestosterone		32	13
Oestradiol-17 β		54	68
Progesterone		100	40
Dexamethasone		100	100
Diethylstilboestrol		100	61

* Methyltrienolone was present at 10nM, triamcinalone acetonide at 5 μM .

dihydrotestosterone and, to a smaller extent, by oestradiol-17 β . Thus, as reported for androgen receptors in other tissues (Barrack & Coffey, 1980), oestrogen can bind to the androgen receptor. On the other hand, progesterone and diethylstilboestrol competed for methyltrienolone binding only at a 1000-fold molar excess, whereas dexamethasone did not compete at all. We therefore conclude that the levator ani muscle contains a cytosolic androgen receptor that is similar in its binding characteristics to the receptor in other tissues.

The data of Fig. 4 show cytosolic androgen receptor binding during various stages of regeneration of the levator ani muscle. It can be seen that after crushing the levator ani muscle, androgen binding decreased by day 2 to about 50% control ($P < 0.01$), and further declined to approx. 25% of control ($P < 0.01$) by day 3. By day 7, however, androgen binding had recovered fully. Binding remained at this level through 30 days after crush. The apparent decline to 85% of the control value at 60 days is not significant.

The cytosol protein concentration of the muscles (mg of protein/g fresh weight) did not change throughout the course of the period after crushing. The respective cytosol protein contents (mg of protein/muscle) on days 2, 3, 7, 14, 30 and 60 were 52, 39, 32, 29, 47 and 61% of control values. The magnitude of these changes corresponded to changes in muscle wet weight after crush and regeneration (results not shown).

Histological observations confirmed previous observations (Gutmann & Carlson, 1978) that the crushing procedure damaged essentially all of the muscle fibres within the levator ani. The first 3 days after crushing constituted the period of breakdown

EXPLANATION OF PLATE 1

Longitudinal section through a levator ani muscle 3 days after crushing

The irregular line (R) is the connective tissue raphe that separates the right and left halves of the muscle. To the right of the raphe, the original muscle fibres have all been broken down, and myoblasts (arrowheads) are the only stages of regeneration represented. To the left of the raphe is a small group of thin, surviving muscle fibres (S). Haematoxylin and eosin stain was used. Magnification 486 $\times$.

EXPLANATION OF PLATE 2

Longitudinal section through a levator ani muscle 30 days after crushing

At this stage the graft is filled with a homogeneous population of cross-striated muscle fibres. Palmgren's silver stain was used. Magnification 486 $\times$.

Fig. 4. Effect of crush lesion and regeneration on specific binding of [^3H]methyltrienolone to the rat levator ani muscle cytosolic receptor

Results are means, with the half-bar representing $\pm$ S.E.M. The numbers of determinations are shown in parentheses. Experimental procedures are described in the text. Filled columns, experimental; open columns, control. *Significantly different from control ($P < 0.01$). The day-3 experimental value was also significantly different ($P < 0.01$) from the experimental values at days 7, 14 and 30, and the day-2 experimental value was significantly different ($P < 0.01$) from the experimental values at days 7, 14, 30 and 60. There were no significant differences between any of the control values.

of original muscle fibres and their removal by macrophages. By day 3 (Plate 1), the phase of breakdown of damaged muscle fibres was complete and most of the muscle consisted of spindle-shaped myoblastic cells occupying the persisting basal laminae of the original muscle fibres. In some scattered areas small clumps of thin multinucleated muscle fibres, possibly representing stages in the repair of less severely damaged muscle fibres, could be seen (Plate 1). By day 7 after crushing, the graft contained a homogeneous population of thin, cross-striated muscle fibres. These fibres continued to mature and to increase in diameter throughout the course of the study (Plate 2).

Discussion

The data provide considerable information concerning the ontogenesis of the cytosolic androgen receptor in the levator ani muscle. This information is important because the levator ani muscle represents an excellent model for studying the effects of sex steroids on 'ordinary' skeletal muscle, which has a much lower level of androgen receptors (Michel & Baulieu, 1980; Krieg *et al.*, 1980; Max, 1981). Furthermore, the levator ani muscle has been shown to be a valuable model tissue for studying the effects of sex steroids on muscle regeneration (Gutmann &

Carlson, 1978; Carlson *et al.*, 1979). The data of Figs. 1–3 and Table 1 show that the levator ani muscle contains a cytosolic androgen receptor that is similar to that found in other androgen target tissues such as prostate gland and kidney (Bullock & Bardin, 1974; Schein *et al.*, 1978; Barrack & Coffey, 1980). The number of receptors found in our determinations is similar to that found for the levator ani-bulbocavernosus complex by Tremblay *et al.* (1977), but much lower than the value reported for these muscles by Krieg (1976). The disparity may be attributable to the experimental procedures employed.

The marked drop in methyltrienolone binding during days 2 and 3 after crushing of the levator ani muscle suggests that there is little, if any, receptor present in the early myoblastic stages of regeneration. Days 2–3 are characterized by the massive breakdown and removal of original muscle cytoplasm, the recruitment and proliferation of a population of myoblastic cells originating, presumably, from satellite cells (Snow, 1977a,b), and the repair of muscle fibres less severely damaged by the crushing procedure (Plate 1). On the basis of the histological findings, the last category of repairing muscle fibres would be the most likely cells to contain the substantial receptor binding remaining after crushing, although the possibility of a low level of receptor binding in mononucleated myoblasts cannot be excluded. The rapid rise in methyltrienolone binding to control values by day 7 may occur after the fusion of myoblasts and the differentiation of myotubes into multinucleated, cross-striated muscle fibres (Plate 2). More exact correlation between receptor binding and specific stages in the differentiation of regenerating muscle fibres requires autoradiographic analysis of the regenerating levator ani muscle. It should be noted further that the hormone-sensitive levator ani muscle is unusual; and, that these results may not be entirely applicable to regeneration of ordinary skeletal muscle.

It is not possible to state precisely whether the marked (4–5-fold) increase in androgen binding between days 3 and 7 represents synthesis of receptor molecules *de novo*. Furthermore, it cannot be stated whether the changes described above represent alterations in receptor number or in receptor affinity, both of which might result in modified hormone binding. The experimental system described herein, however, should be useful for future studies of this phenomenon.

To our knowledge, this is the first study of the development of androgen receptors in muscle. Krieg (1976) and Dubé *et al.* (1976) have indicated that young animals contain greater numbers of receptors in their levator ani muscles than old animals. No studies were performed on embryonic muscle. Such

study would be difficult, *in vivo* or *in vitro*, owing to the small amounts of tissue. The use of regenerating muscle makes such a developmental study feasible.

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