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Suppression of Sodium Channel Function in Differentiating C2 Muscle Cells Stably Overexpressing Rat Androgen Receptors

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Differentiation of skeletal muscle and the formation of the neuromuscular junction are regulated by steroid hormones. The effects of androgens on ion channel proteins central to neuromuscular signalling have been investigated in differentiating mouse muscle C2 cells and in C2 cells that stably overexpress the rat androgen receptor (AR) cDNA. Neither the expression nor function of ACh receptors was regulated by androgenic actions in these cells. However, voltage-dependent sodium (Na) current density was decreased by androgen treatment of C2 cells and was abolished, even in the absence of androgens, in C2 cells that overexpress the AR. The decrease in functional Na current was not accompanied by concomitant decreases in Na channel mRNA, suggesting that AR influence posttranscriptional processing of Na channels in differentiating C2 cells.

[Key words: androgen receptor, sodium channel, ACh receptor, muscle differentiation, patch-clamp recording, gene expression, creatine kinase]

It is well known that androgenic steroids are the determining factor in the induction of the male reproductive system and in the development of masculine secondary sex characteristics. In addition, androgenic steroids play a pivotal role in regulating the plasticity of the brain and the skeletal musculature (for review, see McEwen and Parsons 1982; Erulkar and Wetzel, 1987). Skeletal muscle fibers are one of the primary targets of androgen action outside of the reproductive system (Michel and Baulieu, 1980). Although androgens have anabolic effects on all vertebrate skeletal muscle cells, there are sexually dimorphic muscles that control reproductive behaviors that express significantly higher levels of androgen receptors (ARs) than other skeletal muscles and undergo dramatic changes in response to androgens. The best characterized of these are the levator ani and bulbocavernosus muscles of rodents, the flexor carpi radialis and laryngeal muscles of frogs, and the syringeal muscles of songbirds (for review, see Erulkar and Wetzel, 1987).

The actions of androgenic steroids on sexually dimorphic muscles are diverse, affecting both muscle fiber properties and the formation and function of the neuromuscular junction. Specifically, androgens have been implicated in regulating the contractile properties, as well as the associated expression of metabolic enzymes and myosin isoforms, of both the flexor carpi radialis (Melichna et al., 1972; Rubinstein et al., 1983) and laryngeal (Sassoon et al., 1987; Tobias and Kelley, 1988) muscles of the frog. In addition, androgens have been shown to decrease dye coupling between laryngeal muscle fibers, presumably by decreasing functional gap junctions (Tobias and Kelley, 1988). With regard to neuromuscular transmission, androgens regulate the number of ACh receptors expressed in muscle fibers of the rat levator ani (Bleisch et al., 1982; Bleisch and Harrelson, 1989) and in the syringeal muscle fibers of songbirds (Bleisch et al., 1984), alter the activity of cholinergic enzymes in syringeal motor neurons and muscles (Luine et al., 1980), decrease the expression of calcitonin gene–related peptide in motor neurons innervating the bulbocavernosus muscle in rat (Popper and Micevych, 1990), and modulate ACh receptor function in cultured laryngeal myotubes (Erulkar and Wetzel, 1989). During development, androgens delay the loss of polyneuronal innervation in the levator ani muscle (Jordan et al., 1988, 1989) and produce changes in the morphology of both the pre- and postsynaptic specializations at neuromuscular junctions of bulbocavernosus muscle fibers (Balice-Gordon et al., 1990). Finally, androgens may play a role in modulating voltage-dependent conductances since chronic androgen treatment has been shown to affect the generation of repetitive action potentials in frog layrngeal muscle (Tobias and Kelley, 1988) and to increase the duration of action potentials in the electric organ of electric fish (Mills and Zakon, 1991).

Although the effects of androgens on muscle fiber differentiation and neuromuscular transmission have been well documented, the site of androgen action remains controversial. *In vivo* analysis is complicated by the fact that both sexually dimorphic muscles *and* the motor neurons that innervate them express levels of AR significantly higher than levels expressed in motor neurons and muscle fibers not involved in reproductive behaviors (Arnold et al., 1976; Arnold and Saltiel, 1979; Breedlove and Arnold, 1980, 1983; Kelley, 1980). Therefore, it is not clear *in vivo* whether androgens directly affect the muscle fibers, or if they alter the motor neuron phenotype, which subsequently affects the expression or function of muscle-specific proteins.

In order to study molecular pathways by which androgens affect muscle cell differentiation and subsequent synaptogenesis,

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we have established an androgen-sensitive muscle cell line. We have taken advantage of the mouse muscle C2C12 cell line (C2 cells), which has been shown to undergo most of the developmental changes that characterize maturation of primary muscle cells (Yaffe and Saxel, 1977; Inestrosa et al., 1983; Cox et al., 1991) and are competent to form synaptic specializations when cocultured with cholinergic neurons (Gordon et al., 1993). In the present study, we have developed C2 muscle cell lines that, like sexually dimorphic muscle, stably express high levels of AR. We have characterized the effects of AR on the differentiation of these cells, with particular emphasis on ACh receptors and voltage dependent sodium (Na) channels, two integral membrane proteins that are known to be concentrated at developing neuromuscular synapses and that play essential roles in neuromuscular signaling (Schuetze and Role, 1987; Brehm and Henderson, 1988; Lupa et al., 1993). Here, we report that ARs do not appear to regulate the ability of C2 cells to adopt many of the hallmark properties of differentiated muscle, including myotube formation, increased muscle creatine kinase (mCK) activity, and increased expression of functional ACh receptors. However, the developmental increase in functional Na channel expression is decreased by androgen treatment of parental C2 cells and is abolished in C2 cells that stably overexpress AR.

Materials and Methods

Cell culture conditions. Cultures of the C2C12 mouse skeletal muscle cell line (Yaffe and Saxel, 1977) were grown in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 20% certified fetal bovine serum (GIBCO Laboratories, Grand Island, NY, #240-6000; testosterone levels ~ 0.1 ng/ml as measured by radioimmunoassay) and 0.5% chick embryo extract (GIBCO Laboratories, Grand Island, NY). Fetal bovine serum (FBS) was charcoal stripped prior to adding it to the growth medium; estimated androgen concentrations in this medium were 0.003–0.005 nM. Differentiation was induced by mitogen withdrawal by changing the growth medium ~2 hr after plating the cells to serum-free Opti-MEM I (GIBCO Laboratories), a defined medium that contains no steroid hormones, when cells were ~30% confluent. Data were collected from differentiated, mononucleated myocytes (Caffrey et al., 1989). Although it is possible that this initial and limited exposure to low levels of androgens could initiate a program of differentiation that was then carried out in an androgen-independent manner in steroid-free medium, this possibility seems unlikely given that the perinatal rat brain is sensitive to changes in the steroid environment during a period that lasts 3 weeks (McEwen and Parsons, 1982), suggesting that the critical period of developmental plasticity is much more extensive than a few hours. In addition, for many experiments after initially thawing cells into serum-containing medium, cells were subsequently passaged in serum-containing growth medium that also contained a 100 nM concentration of the anti-androgen, hydroxyflutamide (OHF; SCH 16423: α-α-α-trifluoro-2-methyl-4′-nitro-*m*-lactotoluidide; a gift from the Schering-Plough Corp., Kenilworth, NJ). For experiments using multinucleated myotubes, differentiation was induced by changing the growth medium to DMEM (GIBCO Laboratories) containing 2% charcoal-stripped horse serum (GIBCO Laboratories). Where indicated, the androgenic steroids testosterone (T) and 5-α-dihydrotestosterone (DHT) (Sigma Chemical Corp., St. Louis, MO) were added to media at concentrations of 10 nM. Both androgen-supplemented and unsupplemented Opti-MEM I were exchanged with fresh media every 2 d. To test for the effects of androgen antagonists, cell cultures were maintained for 7 d in Opti-MEM I containing 100 nM hydroxyflutamide.

Transfections. One hundred millimeter plates of C2 cells at ~10% confluence were transfected using the BES variation of the calcium phosphate precipitation method (Chen and Okayama, 1992). Cells were transfected with either 29 μg of a plasmid containing the full-length rat AR cDNA cloned in the pCMV1 expression vector (pCMVrAR; Yarbrough et al., 1989) and 3 μg of the pMAM*neo* expression vector (Clonetech Laboratories, Inc., Palo Alto, CA) or 3 μg of the pMAM*neo* expression vector and 29 μg of salmon sperm DNA (C2*neo* cell lines). After 48 hr, selection for resistance to G418 (400 μg/ml; GIBCO Laboratories) was initiated. G418 concentration was increased to 1 mg/ml after 1 week. After 3 weeks, individual colonies were isolated and propagated as separate clonal cell lines.

Electrophysiological recording and data analysis. All electrophysiological recordings were made at room temperature (22°C) on either an inverted microscope equipped with phase-contrast optics (Zeiss Axiovert 10, MZI Corp., Avon, MA) or an inverted microscope equipped with interference contrast optics (Nikon TMS, Donsanto Corp., Natick, MA). Patch pipettes were fabricated from borosilicate glass (Sutter Instrument Co., Novato, CA) with tip diameters of ~2 μm and resistances of 5–10 MΩ when lightly fire polished and filled. Data were acquired using a List L/M EPC-7 patch-clamp amplifier (Medical Systems Corp., Greenvale, NY) and filtered with the 3 dB point (f_c) set at 10 kHz by an 8-pole Bessel filter (Frequency Devices, Inc., Haverhill, MA). All data were analyzed using an Atari Mega4 computer system and ITC16 computer interface (Instrutech Corp., Elmont, NY).

Single-channel recordings of ACh-induced events were made in the cell-attached configuration of the patch-clamp technique (Hamill et al., 1981) from differentiated mononucleated myocytes. The recording saline contained 140 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES; pH 7.4. Solutions were exchanged several times to ensure that residual androgens from the culture medium were not present in the recording saline. Patch pipettes were filled with 100–300 nM ACh in recording saline and dipped in Sigmacote (Sigma Chemical Corp.) to reduce background noise. Single-channel currents were converted to a video signal by a VR-10B pulse code module (Instrutech Corp.) and stored on videotape for off-line analysis as previously described (Brennan et al., 1992).

Macroscopic voltage-dependent Na currents were recorded in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Data were analyzed from recordings made from mononucleated, differentiated myocytes in order to optimize voltage-clamp control (Caffrey et al., 1989). Recordings were made from myocytes that varied in both shape and size to avoid biasing the sample toward smaller cells, which may have been the ones most recently to cease cell division and begin differentiation. Recordings were also made from multinucleated myotubes in order to ascertain that the absence of Na currents in stably transfected cells was not related to the absence of fusion or to lack of voltage control in the stably transfected myocytes. Quantitative analysis of current density or kinetics was not made from these records due to inadequate voltage clamp and capacity compensation. For whole-cell recordings, culture medium was replaced with recording saline (140 mM Na glutamate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM K glutamate, 10 mM HEPES, 10 mM glucose; pH 7.4). Patch pipettes were filled with 135 mM Cs methanesulfonate, 5 mM CsCl, 10 mM Cs EGTA, 10 mM HEPES; pH 7.4). Voltage command protocols and data analysis were carried out as previously described (Ginty et al., 1992) with the following modifications: cells were held at −90 mV with a 10 msec prepulse to −120 mV prior to each depolarizing test pulse, and then depolarized for 20 msec in 10 mV increments between −60 and +20 mV. Data were digitally filtered at 4 kHz for analysis.

Creatine kinase assay. Creatine phosphokinase activity was measured as described by Massague et al. (1986) using a commercially available kit (Sigma Chemical Corp.). Myoblasts were maintained for 5 d in serum-supplemented medium and myotubes were differentiated in serum-free Opti-MEM I for 5 d. For each determination, two 100 mm plates of myoblasts and two 100 mm plates of myotubes were harvested at 75% confluence in phosphate-buffered saline (PBS) and then freeze-thawed three times in 50 mM glycylglycine, pH 7.4. The cell lysate was collected after pelleting the membrane fraction at 16,000 × *g* for 15 min at 4°C and assayed as described in the kit. Results were normalized to the amount of lysate protein by the method of Lowry et al. (1951).

Cytotoxicity assay. The sensitivity of AR-transfected C2 clones to the Na channel–specific neurotoxin veratridine was assayed according to minor modifications of the procedure of West and Catterall (1979). Triplicate wells of differentiated myocytes grown in 12 well tissue culture trays for 5 d in Opti-MEM I were treated for 36 hr with concentrations of veratridine (Sigma Chemical Corp.; 0.2% ethanol) ranging from 0 to 500 μM. The cells were then washed several times with PBS and cell viability was estimated by determinating the total cellular protein remaining on the plates by the method of Lowry et al. (1951).

ACh receptor binding assay. Acetylcholine receptor number was quantitated by measuring ^{125}I-α-bungarotoxin (^{125}I-α-BTX) binding (Amersham Corp., Arlington Heights, IL) according to Patrick et al. (1977). Cells were grown in triplicate 35 mm plates and differentiated for 5–7 d in Opti-MEM I medium. Because ACh receptor expression in C2 cells is highly dependent on passage number (S. C. Froehner, personal com-

Table 1. ^{3}H-R1881 binding in C2 myotubes and transfected C2 clonal cell lines

	fmol ^{3}H-R1881/ mg protein	% C2 level
Parental C2 cell line		
C2	118 ± 5	100 ± 4
C2 cell lines with elevated levels of AR expression		
CAR2	499 ± 41	423 ± 8
CAR19	359 ± 35	304 ± 10
C2 cell lines with AR levels comparable to parental C2 cells		
CAR30	124 ± 15	105 ± 12
CAR15	105 ± 6	89 ± 6
CAR16	132 ± 11	112 ± 8
C2*Neo*	110 ± 2	93 ± 2
Androgen-sensitive ductus deferens cell line		
DDT_1MF-2	282 ± 34	239 ± 12

Specific binding of the synthetic androgen ^{3}H-R1881 (see Materials and Methods) was assayed to indicate levels of AR expression in parental and stably transfected cell lines. Values represent the means ± SEM of the specific binding for a representative assay measured in triplicate. Comparable results were obtained in 10 separate experiments with different platings of cells, and the relative levels of ^{3}H-R1881 binding in transfected cell lines (compared to parental C2 cells) were similar in all experiments whether binding was normalized to total cellular protein or to DNA. Data from C2 cells are compared with that obtained from the androgen-sensitive DDT_1MF-2 hamster ductus deferens cell line.

munication), all assays were performed on cells at the third passage. After washing the cells in serum-free DMEM, the cells were preincubated for 10 min in DMEM with 0.1% bovine serum albumin (BSA) at 37°C, 5% CO_2. Bungarotoxin binding was initiated by incubating the cells for 1 hr at 37°C, 5% CO_2, in DMEM with 0.1% BSA containing 10 nM ^{125}I-α-BTX. Cultures were then washed three times with ice-cold PBS, cells were scraped off the plates, and ^{125}I-α-BTX binding was measured in a Gamma 4000 counter (Beckman Instruments Inc., Columbia, MD). Nonspecific binding was calculated from parallel plates containing 100 μM *d*-tubocurare in both the preincubation and bungarotoxin binding medium. Toxin binding was normalized to total cellular protein (Lowry et al., 1951).

Androgen receptor binding assays. Total AR binding was quantitated by minor modifications of the procedures described by Syms et al. (1987) and Kemppainen et al. (1992). Triplicate cultures of 35 or 60 mm dishes were differentiated in Opti-MEM I for 5–7 d. The assay was initiated by incubating the cultures for 60 min at 37°C, 5% CO_2 in DMEM containing 2 or 5 nM ^{3}H-methyltrienolone (^{3}H-R1881; Du Pont New England Nuclear Research Products, Boston, MA), a synthetic ligand for the AR. The assay mixture also included 0.1 μM cortisol and 1 μM triamcinolone acetonide to reduce ^{3}H-R1881 binding to corticosterone and progesterone receptors, respectively (Bernard et al., 1984). Nonspecific binding was measured by the addition of either a 100-fold or a 2000-fold excess of nonradioactive R1881 in parallel dishes. No significant differences in estimates of total binding were obtained by these variations in the concentration of ^{3}H-R1881 or unlabeled R1881. Cultures were washed three times with PBS, cells were harvested, and the radioactivity extracted overnight with ethanol at 4°C. Steroid binding was quantitated by liquid scintillation counting, and normalized to cellular protein (Lowry et al., 1951).

Preparation of RNA and Northern blot analysis. RNA was isolated from cells plated in 100 mm tissue culture dishes and maintained in Opti-MEM I for 5–7 d. Total cellular RNA was isolated according to Chirgwin et al. (1979) from cultures at ~50–80% confluence; 40 μg samples of RNA were size fractionated on 0.8% agarose gels containing 2.0 M formaldehyde. After electrophoresis, RNA was transferred to a nylon filter (Zetabind, Cuno, Inc., Meridan, CT) overnight, and the filter was baked at 80°C under vacuum for 2 hr. Complementary RNA probes were generated using the Gemini II kit (Promega Corp., Madison, WI) and a cDNA template encoding a portion of the rat brain type II α subunit of the Na channel, which has been used in previous studies to detect the ~8.5 kilobase (kb) muscle Na channel transcripts (Cooperman et al., 1987; Trimmer et al., 1989). The specific activity of the probes was 1–2.5 × 10^8 dpm/μg. Filters were prehybridized for 4–5 hr at 62°C in 50% formamide, 0.1% SDS, 0.1% ficoll, 0.1% polyvinylpyrolidine, 0.1% BSA, 5× SSPE (150 mM NaCl, 1 mM EDTA, 10 mM NaH_2PO_4; pH 7), 100 μg/ml denatured salmon sperm DNA, and were then hybridized for 36–40 hr at 62°C in 50% formamide, 0.1% SDS, 0.04% ficoll, 0.04% polyvinylpyrolidine, 0.04% BSA, 5× SSPE, 100 μg/ml denatured salmon sperm DNA, 10% dextran sulfate containing the RNA probe at a final concentration of 4 × 10^6 dpm/ml. After hybridization, the filters were washed twice at room temperature in 2× SSPE, 0.1% SDS (15 min each); once at room temperature in 0.1× SSPE, 0.1% SDS (15 min); twice at 59°C in 0.1× SSPE, 0.1% SDS (30 min each); and were autoradiographed on Kodak X-OMAT AR film (Rochester, NY). Autoradiographs were scanned and signals quantitated using NIH IMAGE software. As an internal control for variations in the amount of RNA loaded in each lane of the gel, signals for Na channel mRNA were normalized to ethidium bromide staining of 18S ribosomal RNA.

All values indicate means ± standard errors of the mean (SEM). Statistical significance was determined using a Student's two-tailed *t* test.

Results

Generation of C2 muscle cell lines overexpressing androgen receptors

C2 myoblasts were cotransfected with a plasmid containing a full length cDNA encoding the rat androgen receptor (pCMVrAR) and a plasmid containing a gene conferring resistance to neomycin (pMAM*neo*). Previous studies have shown that receptors expressed from pCMVrAR in mammalian cells appear identical to native receptors (Quarmby et al., 1990). Stably transfected cells were selected in neomycin-supplemented medium and individual cells propagated as separate clonal cell lines. AR expression in C2 cells and 23 of the stably transfected clonal cell lines was assayed by specific binding of the synthetic ligand ^{3}H-R1881 to cytosolic AR in differentiated myotubes. Appreciable ^{3}H-R1881 binding was evident in untransfected C2 cells (Table 1). Four of the clonal C2 cell lines stably transfected with the AR cDNA demonstrated a three- to fivefold increase in ^{3}H-R1881 binding when compared to parental C2 cells. Although the levels of AR are higher in untransfected C2 cells than in adult primary muscle fibers, the ratio of AR expressed in transfected versus parental C2 cells is similar to the ~5–10-fold higher levels of AR expressed in sexually dimorphic versus nondimorphic primary muscles (for review, see Erulkar and Wetzel, 1987). Two of these lines (CAR2 and CAR19) with high levels of AR expression (Table 1) were selected for further analysis. To control for effects of transfection not directly related to overexpression of AR, data were also analyzed from a clonal cell line stably transfected with the pMAM*neo* plasmid alone (C2*neo*) and from three clonal cell lines (CAR15, CAR16, and CAR30), which were transfected with both pCMVrAR and pMAM*neo*, but nonetheless expressed ^{3}H-R1881 binding at levels comparable to those of the parental C2 cells (Table 1).

Morphological characterization and differentiation of C2 cells overexpressing androgen receptors

Undifferentiated C2 myoblasts exhibit a spindle-shaped morphology when grown in the presence of serum-supplemented media (Fig. 1*A*). Expression of pCMVrAR and pMAM*neo* in C2 cells did not lead to appreciable alterations in myoblast morphology under these conditions (Fig. 1*B*). When the serum concentration in the culture medium is reduced, C2 myoblasts cease cell division and fuse to form multinucleated myotubes. The overexpression of AR in the CAR2 and CAR19 cells did not inhibit their ability to form myotubes upon serum withdrawal. In fact, myotubes formed in these overexpressing lines were often larger and more robust in appearance than those

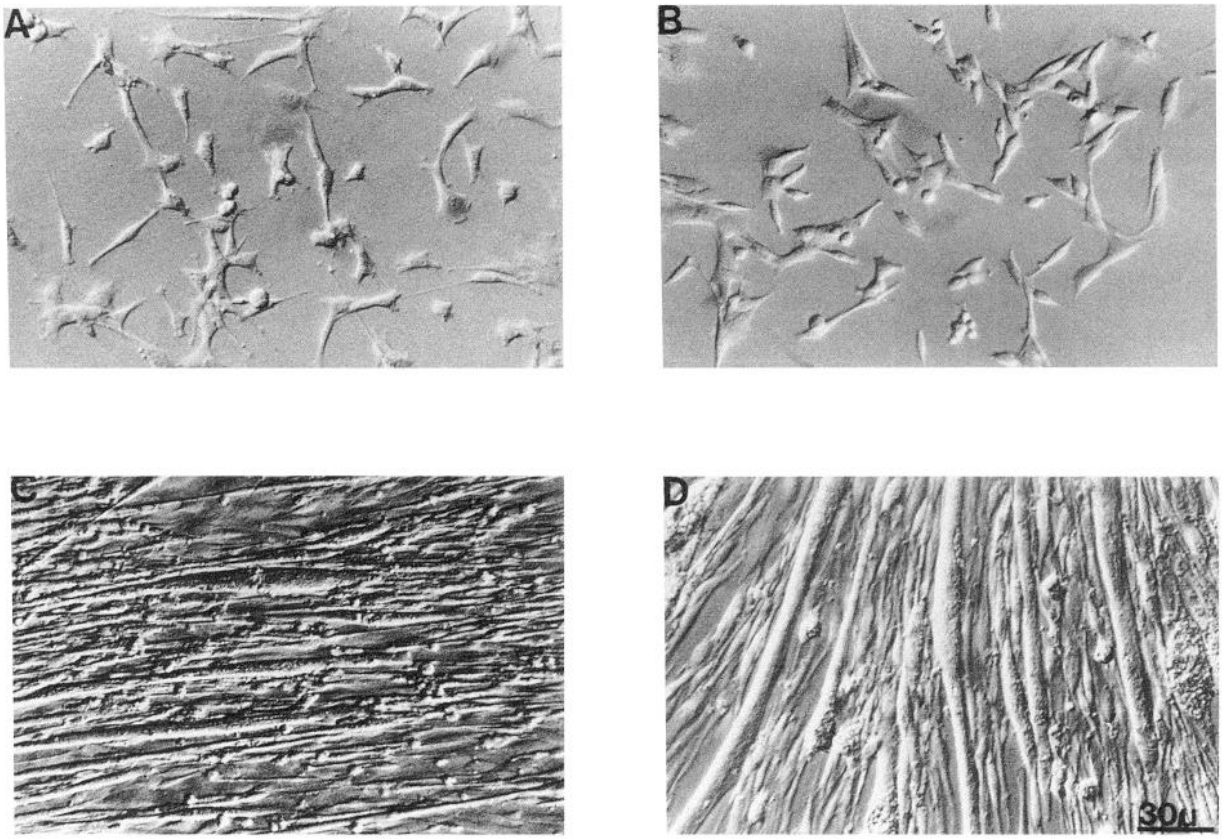

Figure 1. Morphology of C2 and CAR2 myoblasts and myotubes. Photomicrographs of cells viewed under interference contrast optics illustrating the similarity in morphology of C2 myoblasts (*A*) and CAR2 myoblasts (*B*) in the log phase of growth and multinucleated myotubes of C2 cells (*C*) and CAR2 cells (*D*) maintained for 7 d in serum-depleted medium.

derived from the parental C2 cells (compare Fig. 1, *C* and *D*). Neither C2, CAR2, nor CAR19 cells demonstrated dramatic differences in their ability to form multinucleated myotubes when differentiated for 7 d in medium supplemented with 10 nM testosterone and 10 nM 5-α-dihydrotestosterone (T/DHT).

Concomitant with fusion, muscle cell differentiation leads to the increased expression of a number of muscle-specific genes, including those encoding mCK, α-actin, myosin heavy chain (for review, see Rosenthal, 1989), nicotinic ACh receptors (for review, Schuetze and Role, 1987; Brehm and Henderson, 1988), and voltage-dependent Na channels (for review, Mandel, 1992). To assess further whether expression of pCMVrAR or pMAM*neo* disrupted the ability of C2 cells to undergo differentiation, mCK activity was assayed in proliferating myoblasts and differentiated myocytes of parental C2 cells and stably transfected C2 cell lines. Although there was variability in the absolute levels of mCK activity observed among the individual cell lines and among different platings of cells, all of the stably transfected cell lines exhibited increases in mCK activity upon serum withdrawal that were as great as those observed in parental C2 cells (Fig. 2). These data, coupled with the ability of cells to form multinucleated myotubes, demonstrate that expression of pCMVrAR or pMAM*neo* does not completely prevent the differentiation of C2 cells.

ACh receptor expression and function

Increased expression of nicotinic ACh receptors is a hallmark of differentiation for all skeletal muscle cells and essential for

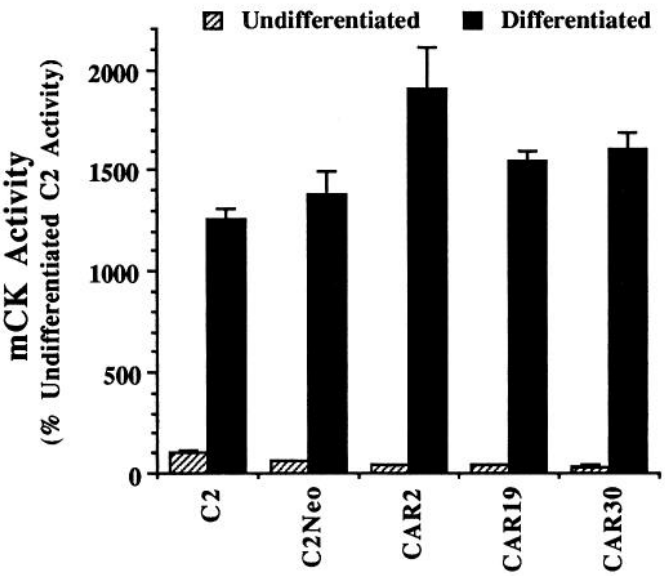

Figure 2. Differentiation-induced increases in muscle creatine kinase (*mCK*) activity in C2 cells and stably transfected C2 cells. mCK activity was assayed in parental C2 cells, transfected cells with elevated AR expression (CAR2 and CAR19), and transfected cells with levels of AR comparable to parental C2 cells (CAR30 and C2*neo*). mCK activity was assayed for myoblasts maintained for 5 d in medium containing 20% fetal bovine serum (*Undifferentiated*) and for myocytes (*Differentiated*) maintained for 5 d in serum-free medium. Data was normalized to the levels of mCK activity in C2 myoblasts. Values represent the means ± SEM for two separate platings of cells assayed in triplicate.

Table 2. ^{125}I-α-BTX binding in C2 myotubes

	pmol α-BTX/mg protein	
	− Androgens	+ Androgens
Parental C2 cell line		
C2	1.00 ± 0.05	1.06 ± 0.14
Cell lines with elevated levels of AR expression		
CAR19	1.01 ± 0.11	1.08 ± 0.08
CAR2	1.13 ± 0.08	1.03 ± 0.08
Cell lines with AR levels comparable to parental C2 cells		
CAR30	0.92 ± 0.05	1.18 ± 0.03
C2*neo*	0.90 ± 0.01	0.88 ± 0.01

Levels of ACh receptor expression in differentiated myotubes were measured by assaying binding of 10 nM ^{125}I-α-BTX to intact cells. Nonspecific binding was measured in presence of 100 μM *d*-tubocurare; 10 nM T and 10 nM DHT were added to the medium for 7 d (+Androgens). Values represent the means ± SEM of specific binding for a representative assay performed in triplicate. Comparable results were obtained in five separate experiments with different platings of cells.

Table 3. Properties of ACh-induced single channel events in differentiated C2 myocytes

	γ (pS)	τ_1 (msec)	τ_2 (msec)
Parental C2 cell line			
C2	52.3 ± 0.9	0.30 ± 0.06	4.41 ± 0.46
	(*n* = 17)	(*n* = 18)	(*n* = 18)
C2/T/DHT	53.1 ± 2.3	0.23 ± 0.03	4.27 ± 0.42
	(*n* = 14)	(*n* = 17)	(*n* = 17)
Cell lines with elevated levels of AR expression			
CAR2/CAR19	54.2 ± 1.7	0.28 ± 0.03	4.80 ± 0.21
	(*n* = 20)	(*n* = 24)	(*n* = 24)
CAR2/19/T/DHT	50.6 ± 1.0	0.37 ± 0.08	5.56 ± 0.43
	(*n* = 24)	(*n* = 32)	(*n* = 32)

Values indicate single channel slope conductances (γ) and the time constants (τ_1 and τ_2) estimated for a biexponential distribution of mean open durations for ACh-induced single-channel events recorded in the cell-attached configuration from differentiated myocytes maintained in steroid-free medium or medium supplemented with T/DHT for 7 d. Values represent means ± SEM for the number of cells indicated in parentheses. Total membrane potential was approximately −120 mV; [ACh] = 250 nM.

neuromuscular transmission (Schuetze and Role, 1987; Brehm and Henderson, 1988). Androgens have been shown to increase the number of ACh receptors expressed in sexually dimorphic muscles (Bleisch et al., 1982, 1984; Bleisch and Harrelson, 1989). To determine whether the differentiation-induced increase in ACh receptor expression occurred in C2 cells overexpressing AR and to assess the effects of androgens on ACh receptor expression during differentiation of both parental C2 cells and C2 cell lines overexpressing AR, ACh receptor expression was measured by ^{125}I-α-BTX binding. Upon differentiation induced by serum-free and steroid-free medium, levels of ACh receptors expressed in the transfected cell lines were indistinguishable from the parental C2 cells and comparable to those previously reported for differentiated C2 cells (LaRochelle et al., 1989) (Table 2). These data further suggest that the increased expression of AR does not block differentiation of C2 cells.

To test whether androgen treatment led to increased expression of ACh receptors, differentiated cultures of either C2 cells or C2 cells overexpressing the AR were maintained for 7 d in medium supplemented with T/DHT. In contrast to previous studies that demonstrated that androgens increased ACh receptor expression *in vivo* (Bleisch et al., 1982, 1984; Bleisch and Harrelson, 1989), growing cells in androgen-supplemented medium did not lead to significantly higher levels of ^{125}I-α-BTX binding in either differentiated C2 cells or in differentiated C2 cells overexpressing AR (Table 2).

To assess whether the physiological properties of ACh receptors were altered by overexpression of AR or by androgen treatment, single-channel ACh-induced events were analyzed from cell-attached patches of membrane from C2, CAR19, or CAR2 differentiated myocytes. The predominant class of ACh-induced events recorded from myocytes of these cell lines had single-channel properties characteristic of the embryonic class of ACh receptor (Brehm and Henderson, 1988; Table 3) previously shown to be expressed in C2 cells (Martinou and Merlie, 1991). Neither overexpression of AR nor differentiation of the cells in steroid-supplemented media for 7 d led to significant differences in single-channel slope conductance or open time kinetics of ACh receptors expressed in C2, CAR2, or CAR19 cell lines (Table 3).

Since levels of testosterone as low as 0.001 nM have been shown to activate androgen-sensitive genes in transiently transfected cell lines (Kemppainen et al., 1992), it was possible that residual androgenic steroids from the serum in the growth medium were inducing androgen-dependent changes in ACh receptor expression in cultures differentiated in steroid-free medium. To address this possibility, C2, CAR2, and CAR19 cells were maintained continuously in the presence of saturating concentrations (100 nM) of OHF, an anti-androgen that specifically binds to AR, but does not cause transcriptional activation of AR-sensitive genes (Kemppainen et al., 1992). The functional properties of the ACh receptors expressed in these cells maintained in OHF were similar to those maintained in its absence (data not shown). Taken together, the results from both ^{125}I-α-BTX binding assays and single-channel analysis suggest that ACh receptor expression in differentiating C2 cells is not altered by the overexpression of the AR or by androgen treatment.

Pharmacological assay of voltage-dependent Na channel expression

The expression of voltage-dependent Na channels is regulated during development of skeletal muscle cells *in vivo* (Weiss and Horn, 1986; Kallen et al., 1990; Trimmer et al., 1990) and in differentiating muscle cell lines (Caffrey et al., 1987, 1989). To determine if overexpression of AR led to changes in Na channel expression during differentiation, parental C2 cells, CAR2, CAR19, C2*neo*, and CAR30 cells were incubated in steroid-free medium supplemented with veratridine, a lipid-soluble neurotoxin that binds specifically to the Na channel and shifts the voltage dependence of Na channel activation so that most channels are open at the resting membrane potential (for review, see Catterall, 1992). Because prolonged activation of Na channels ultimately results in cell death, survival rates of cells after prolonged exposure to veratridine can be used as an indication of the levels of functional Na channels in the cell membrane (West and Catterall, 1979; Wu and Poo, 1983). After a 36 hr incubation in steroid- and serum-free medium containing 500 μM veratridine, only 5% of C2 cells and stably transfected C2 cell lines (C2*neo* and CAR30) that have AR expression comparable to parental C2 cells remain viable (Fig. 3). Similar results were obtained in two other clonal cell lines (CAR15 and CAR16) that did not have elevated levels of AR expression (data not shown). Surprisingly, greater than 80% of the CAR2 and CAR19

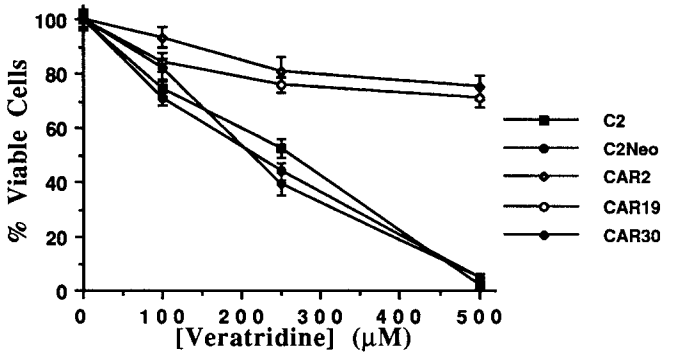

Figure 3. Veratridine-induced cytotoxicity in C2 cells and stably transfected C2 cell lines. The viability of differentiated myocytes (maintained for 7 d in steroid-free medium) of parental C2 cells, transfected cells with elevated AR expression (CAR2 and CAR19), and transfected cells with levels of AR comparable to parental C2 cells (CAR30 and C2*neo*). The percentage of nonviable cells was estimated by quantitating the protein remaining on the plates after 36 hr at the concentration of veratridine indicated on the *abscissa*. Values represent the means ± SEM for a representative assay performed in triplicate. Comparable results were obtained in three separate experiments with different platings of cells.

cells (which have increased levels of AR) remained viable after 36 hr (Fig. 3). The effects of veratridine treatment did not appear to depend on androgen binding to its cognate receptor since there was no significant change in the veratridine sensitivity of any of the cell lines when cultures were differentiated in the presence of the androgens, T/DHT, or in the presence of the anti-androgen, OHF (data not shown).

Electrophysiological analysis of voltage-dependent Na channels

The functional properties of voltage-dependent Na channels were assessed in differentiated myocytes from C2, CAR2, CAR19, CAR30, and C2*neo* cell lines using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Ninety-three percent of C2 cells maintained in steroid-free medium exhibited large (average peak amplitude > 1 nA) voltage-dependent Na currents (Fig. 4, Table 4). The peak Na current in C2 myocytes occurred at potentials between −30 and −20 mV and reached its maximum approximately 1 msec after the onset of depolarization. Estimates of cell membrane area, made from measurements of cell membrane capacitance, were used to calculate Na current densities (Table 4).

Maintaining differentiated C2 myocytes in medium supplemented with T/DHT for 7 d significantly reduced the average Na current density to 50% of that for C2 cells grown in steroid-free medium ($p < 0.01$; Table 4), but did not appreciably alter the kinetics of the macroscopic Na currents (data not shown). Maintenance of differentiated C2 myocytes for 7 d in medium supplemented with T/DHT *and* saturating concentrations of OHF blocked the androgen-induced decrease in Na current density (Table 4). The decrease in current density could not be attributed to a block of the channels by residual androgens from the culture medium since culture dishes were washed several times with saline prior to recording and, in separate experiments, acute addition of 10 nM T/10 nM DHT to the recording saline had no significant effect on the functional Na current density or the kinetics of the Na currents recorded from C2 myocytes (data not shown).

The cytotoxicity assays with veratridine suggest that differentiated CAR2 and CAR19 myocytes, even when maintained in steroid-free medium, express fewer functional Na channels than parental C2 cells or channels with significantly altered single channel properties. Whole-cell recordings made from CAR2 and CAR19 cells dramatically confirmed this hypothesis. In no case ($n = 78$) were detectable voltage-dependent Na currents (>10 pA) elicited in either CAR2 or CAR19 differentiated myocytes (Fig. 4, Table 4). Differences in steady state Na channel inactivation arising from differences in cell size and inadequate voltage control in the larger CAR2 and CAR19 cells could not account for the inability to elicit Na currents in these cells. In

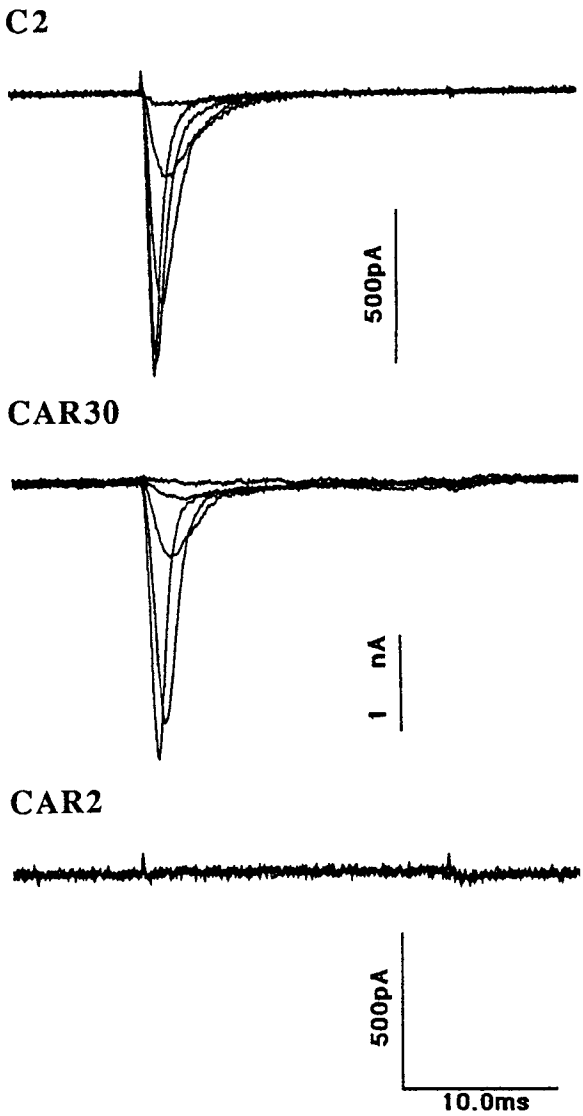

Figure 4. Macroscopic Na currents in C2 and stably transfected C2 cells: representative Na current records elicited in the whole-cell configuration of the patch-clamp technique from cultures maintained in serum- and steroid-free medium for 7 d of parental C2 myocytes (*top*); CAR30 myocytes, stably transfected cells that do not over express AR (*middle*); and CAR2 myocytes, stably transfected cells that express elevated levels of AR (*bottom*). Recordings were made under ionic conditions designed to isolate Na currents (see Materials and Methods). Shown for each cell are five superimposed current records elicited by 20 msec step depolarizations from a holding potential of −90 mV to −60, −50, −40, −30, and −20 mV. Currents shown for this figure were filtered at 2 kHz with capacitance and leakage currents digitally subtracted from the records using a scaled pulse ($-P/4$) routine.

Table 4. Properties of voltage-dependent Na currents in differentiated C2 myocytes

	Fraction of cells with I_{Na}	Peak I_{Na} (nA)	Capacitance (pF)	Density (nA/pF)
Parental cell line				
C2	28/30 (93%)	1.18 ± 0.14	12.7 ± 0.8	0.10 ± 0.01
C2/T/DHT	21/26 (81%)	0.53 ± 0.10	11.2 ± 1.1	0.06 ± 0.01*
C2/T/DHT/OHF	34/40 (85%)	1.55 ± 0.22	13.6 ± 0.9	0.13 ± 0.02
pCMVrAR transfected cells				
High levels of ^{3}H-R1881 binding				
CAR 19	0/24 (0%)	ND	17.8 ± 1.0	ND
CAR 2	0/54 (0%)	ND	28.6 ± 2.1	ND
Parental levels of ^{3}H-R1881 binding				
CAR 30	12/14 (86%)	1.74 ± 0.56	49.6 ± 6.6	0.04 ± 0.01
pMAM*neo* transfected cells				
C2*neo*	19/22 (86%)	1.92 ± 0.29	34.8 ± 2.0	0.06 ± 0.01

Data show analysis of voltage-dependent Na currents recorded in the whole-cell configuration of the patch-clamp technique from differentiated myocytes maintained for 7 d in serum- and steroid-free medium. T/DHT were added to the medium at either 1 nM (21 cells) or 10 nM (19 cells); since no significant differences were evident between the two concentrations, data have been pooled. OHF was added to the medium at 100 nM. The level of current detection was 10 pA. Values represent means ± SEM for the number of cells with detectable I_{Na}, as indicated in left column.

* $p < 0.01$; ND indicates not detectable.

recordings made from large, fused myotubes in both C2 and CAR2 cultures, voltage-dependent Na currents, although poorly clamped, were elicited in nearly all fused, multinucleated myotubes in C2 cultures, but were not discernible in recordings made from any CAR2 myotubes (data not shown), suggesting that differences in size alone cannot account for the absence of current in the CAR2 and CAR19 myocytes. Nor could the dramatic difference in Na current density in cells overexpressing AR be attributed to a nonspecific effect of transfection or antibiotic resistance, since in stark contrast to CAR2 and CAR19 cells, large voltage-dependent Na currents ($I_{avg} > 2$ nA) with activation properties similar to parental C2 cells were elicited in greater than 80% of differentiated myocytes from both the CAR30 and the C2*neo* cell lines (Fig. 4, Table 4). Sodium currents were also elicited from differentiated myocytes of the CAR15 and CAR16 cell lines, which are transfected, but do not express elevated levels of AR (data not shown). Although both CAR30 and C2*neo* myocytes were substantially larger than CAR2 and CAR19 cells (Table 4), voltage-dependent Na currents were nonetheless readily elicited in these cells, again suggesting that a size-dependent lack of voltage control cannot account for the absence of currents in the CAR2 and CAR19 myocytes. As with voltage-dependent currents elicited in C2 cells, currents recorded from CAR30 cells were reversibly blocked by substitution of Tris for Na and were blocked by TTX (data not shown). The finding that depolarization could elicit macroscopic Na currents in C2, CAR30, CAR15, CAR16, and C2*neo* myocytes, but not in CAR2 or CAR19 myocytes is consistent with the reduced veratridine sensitivity observed in the latter two cell lines, and suggests that the absence of functional Na currents in CAR2 and CAR19 cell lines is directly correlated with increased expression of AR. Of particular interest was the finding that neither incubation in T/DHT-supplemented medium nor in medium supplemented with saturating concentrations of OHF led to the detection of any voltage-dependent Na currents in CAR2 or CAR19 cells, suggesting that the absence of functional Na current in CAR2 and CAR19 cells does not depend on androgen binding to its cognate receptor.

Northern blot analysis of Na channel gene expression

The decrease in Na current density in C2 cells treated with androgens and in untreated C2 cell lines expressing increased levels of AR could be produced by a number of disparate cellular mechanisms. To determine whether the decrease in Na current is correlated with changes in Na channel gene expression, steady state levels of Na channel α-subunit mRNA were analyzed by Northern blot hybridization. A ^{32}P-labeled probe was generated using a portion of the coding region of the rat brain type II Na channel α-subunit gene, which, at moderate stringency, has been shown to cross-hybridize with mRNAs encoding muscle Na channel α-subunits (Cooperman et al., 1987). This probe detected a transcript of approximately 8.5 kb in RNA isolated from the various C2 cell lines (Fig. 5), consistent with the size of rat skeletal muscle Na channel α-subunit mRNAs previously reported (Kallen et al., 1990). RNA was isolated from C2 cells maintained for 7 d in medium supplemented with T/DHT, in medium supplemented with T/DHT *and* saturating concentrations of OHF (T/DHT/OHF), or in medium supplemented with OHF alone. Steady state levels of Na channel mRNA were quantitated by densitometry of the Na channel mRNA signal in autoradiograms and normalization of these signals to that corresponding to 18S RNA for each sample. Although the treatment with androgen-supplemented medium led to a 50% decrease in Na current density, steady state levels of Na channel α-subunit mRNA in T/DHT-treated cultures of C2 cells were not lower than the levels of Na channel α-subunit mRNA observed in untreated C2 cells (Fig. 5). As expected from this result, neither treatment with T/DHT/OHF nor with OHF alone led to appreciable changes in the steady state levels of Na channel α-subunit mRNA in C2 cells (Fig. 5). Consistent results were obtained with RNA isolated from three separate platings of cells.

The steady state levels of Na channel α-subunit mRNA were also determined for CAR19, CAR2, CAR30, and C2*neo* cell lines maintained in steroid-free medium. Although voltage-dependent Na currents and veratridine-induced cell death were not observed in the CAR2 and CAR19 cultures, Na channel α-

subunit mRNA was readily detectable in these lines at steady state levels comparable to those detected in parental C2, CAR30, and C2*neo* cell lines (Fig. 5). Consistent results were obtained with RNA isolated from three separate platings of cells. Taken together, these results suggest that neither androgen treatment of C2 cells nor overexpression of the AR led to significant changes in Na channel gene expression.

Discussion

Androgenic steroids play a pivotal role in modulating both neuromuscular junction formation and muscle fiber phenotype in sexually dimorphic muscles. As an avenue toward understanding the molecular mechanisms by which androgens affect muscle cell differentiation and subsequent synapse formation, we have developed C2 muscle cell lines that stably overexpress AR and have investigated the effects of androgenic steroids on the properties of both parental C2 cells and C2 cells overexpressing this steroid receptor. We find that although androgens are present in serum, they do not appear to play a critical role in the ability of C2 cells to initiate the program of differentiation. When differentiation is initiated in these cell lines by depleting the medium of serum, both parental C2 cells and the C2 cell lines overexpressing AR ceased cell division, fused to form multinucleated myotubes, and increased their expression of muscle specific proteins, such as mCK and ACh receptors. These developmental events occurred for all cell lines whether they were differentiated in androgen-supplemented medium, in steroid-free medium, or in medium in which androgenic actions were blocked by saturating concentrations of OHF.

One facet of muscle cell differentiation that is critical for synaptogenesis and efficient neuromuscular transmission is ACh receptor expression. Androgens have been reported to increase the levels of ACh receptors in sexually dimorphic muscles of rats and songbirds (Bleisch et al., 1982, 1984; Bleisch and Harrelson, 1989). Our results demonstrate that overexpression of AR did not inhibit ACh receptor expression or function in C2 cells, consistent with the ability of AR-overexpressing cells to fuse and increase mCK activity during differentiation. However, contrary to the studies on androgen regulation of ACh receptor expression *in vivo,* we found that neither androgen treatment nor overexpression of the AR *increased* ACh receptor expression in these cells. The difference in androgen-dependent effects on muscle ACh receptor expression *in vivo* versus cultured C2 cells could arise if androgens affect motor neurons which in turn alter ACh receptor expression in sexually dimorphic muscle. Similarly, since C2 cells are both embryonic and uninnervated, two conditions that promote high levels of ACh receptor expression (Henderson and Brehm, 1988), it is possible that the levels of ACh receptor in C2 cells are already near maximal and that androgen treatment cannot further increase the number of these receptors in the cell membrane. These possibilities could be tested in future experiments by determining the effects of androgens in cocultures of C2 cells innervated with primary motor neurons or under conditions where direct electrical stimulation significantly decreases ACh receptor expression. Alternatively, it is clear that activated steroid–receptor complexes do not regulate gene expression in isolation; steroid responsiveness depends not only upon the presence of steroid receptors and genes with hormone response elements, but on the intracellular milieu of transcription factors, which may vary significantly in a cell-specific manner (S. Adler et al., 1988; Schule et al., 1990; Yang-Yen et al., 1990; A. J. Adler et al., 1992; Gronemeyer, 1992; Pearce and Yamamoto, 1993). Although the C2 cell lines express AR, specific factors necessary for androgen-dependent transcriptional regulation *in vivo* may not be expressed in these cell lines at levels required for optimal activation of androgen-sensitive genes and increased ACh receptor expression.

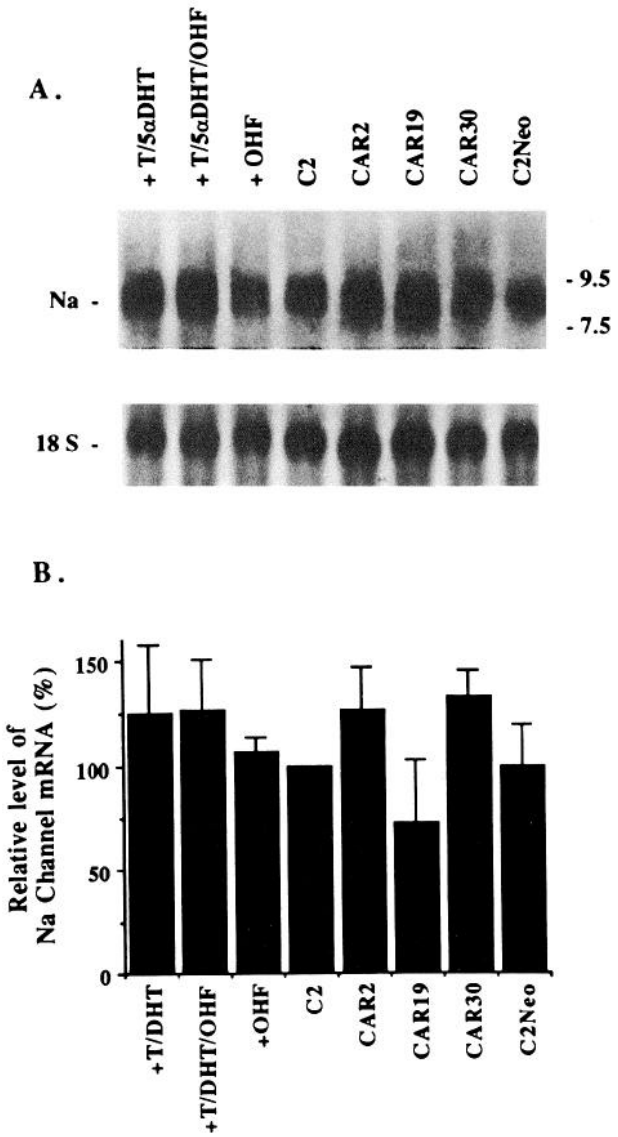

Figure 5. Northern blot analysis of Na channel mRNA in C2 cells and stably transfected C2 cell lines. *A, From left to right,* Na channel mRNA (*Na*) and ribosomal RNA (*18 S*) in differentiated C2 cells maintained in steroid-supplemented medium (*+T/5αDHT*), C2 cells maintained in medium supplemented with steroids and hydroxyflutamide (*+T/DHT/OHF*), C2 cells maintained in medium supplemented with hydroxyflutamide (*+OHF*), C2 cells in unsupplemented Opti-MEM I, CAR2 cells, CAR19 cells, CAR30 cells, and C2*neo* cells. Positions of commercially available RNA molecular weight standards are indicated on the *right.* For Na channel mRNA, the autoradiogram signal shown was quantitated by densitometry. For the 18S ribosomal RNA, the bottom portion of the gel was stained with ethidium bromide and photographed. The negative of the photograph was then quantitated by densitometry. *B,* For each of the cell types and conditions shown in *A,* the level of Na channel mRNA was normalized to 18S RNA and then expressed as the percentage of Na channel mRNA in parental C2 cells maintained in serum-free medium (C2 = 100%). Data represent the means ± SEM from three separate platings of cells.

In contrast to the absence of androgen-dependent effects on

ACh receptor levels, functional Na channel expression was significantly altered by androgenic actions in both C2 cells and C2 cells overexpressing AR. In parental C2 cells, the average whole-cell Na current density recorded from myocytes grown in androgen-supplemented medium was half that for cells grown in steroid-free medium. The decrease in Na current density elicited by treatment with T/DHT was specific to activation of the AR since it was blocked by addition of saturating concentrations of OHF. To assess whether the androgen-induced decrease in Na current density could be attributed to a decrease in Na channel α-subunit gene expression, the steady state levels of Na channel α-subunit mRNA were determined by Northern blot analysis for untreated and androgen-treated C2 cells. Densitometric analysis of autoradiograms indicated that the androgen-induced decrease in Na current density was not associated with a concomitant decrease in the steady state levels of Na channel α-subunit mRNA, and the androgen-dependent reduction in Na current density in C2 cells may arise from posttranscriptional changes in Na channel expression.

Overexpression of the AR was correlated with the complete absence of macroscopic Na current in differentiating C2 cells. We did not detect voltage-dependent Na currents in *any* myocytes from the CAR2 and CAR19 lines which overexpress AR. In contrast, robust Na currents were elicited in almost all myocytes from the C2*neo,* as well as the CAR15, CAR16, and CAR30 cell lines, which were transfected with the AR cDNA but express AR at levels comparable to parental C2 cells. Quite surprising was the observation that suppression of Na current in the CAR2 and CAR19 cell lines occurred in steroid-free medium. Since we were able to elicit voltage-dependent Na currents in four separate clonal cell lines that were transfected, but did not overexpress the AR, it seems unlikely that the suppression of Na current was a result of the transfection procedure itself. Rather, we believe increased levels of the AR, in the absence of ligand binding, lead to the decrease in Na current density in the CAR2 and CAR19 cell lines. A number of recent reports have shown that ligand-free hormone receptors can have profound effects on gene expression and differentiation by interfering with the actions of other ligand-bound nuclear receptors (Damm et al., 1989; Munoz et al., 1993) or by altering signaling through second messenger pathways (Denner, 1990; Power et al., 1991, 1992). Although we do not know the mechanisms by which AR interfere with Na channel function in C2 cells, Northern blot analysis indicates that steady state levels of Na channel α-subunit mRNA in CAR2 and CAR19 cell lines were comparable to those in parental C2, C2*neo,* and CAR30 cells. Therefore, it does not appear that overexpression of the AR affects expression of the Na channel α subunit gene. However, modulation of Na channel assembly and function by posttranscriptional mechanisms has been well documented (for review, see Catterall, 1992), and it is possible that overexpression of the AR alters constituents of intracellular signaling pathways necessary for functional Na channel expression. The deduced amino acid sequence of both the adult (SkM1) and embryonic (SkM2) forms of the muscle Na channel α subunits contain consensus sites for phosphorylation by protein kinase A (PKA), as well as protein kinase C (PKC) and casein kinase II (Trimmer et al., 1989; Kallen et al., 1990), and the α subunit is phosphorylated by PKA in intact cultured muscle cells (Yang and Barchi, 1990). Changes in PKA activity have been shown to alter the functional properties of neuronal (Gershon et al., 1992; Ginty et al., 1992; Li et al., 1992; Smith and Goldin, 1992) and cardiac Na channels (Ono et al., 1989; Schubert et al., 1989). Although the effects of phosphorylation on Na channel function have not been examined in skeletal muscle, these channels may be identical to those in cardiac muscle (Gellens et al., 1992), and in preliminary experiments we have found that a 24 hr incubation of 2-d-old cultures of C2 myocytes with a 100 μM concentration of the permeant cAMP analog 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (8-CPT-cAMP) led to a 48% reduction in peak Na current density (34.4 ± 12.4 pA, $n = 8$, vs 18.0 ± 3.3 pA, $n = 10$), similar to the results obtained with cAMP treatment of cardiac myocytes (Ono et al., 1989; Schubert et al., 1989).

A number of other potential posttranslational mechanisms could also account for the AR-dependent decrease in functional Na current observed in C2 cells. For example, stable expression of the activated form of c-H-*ras* has been shown to result in selective suppression of voltage-dependent Na and calcium currents in differentiating BC_3H1 muscle cells (Caffrey et al., 1987) and suppression of TTX-insensitive Na currents in the AtT-20 pituitary cell line (Flamm et al., 1990). Furthermore, androgens have been shown to increase levels of protooncogenic *ras* mRNA in prostate cells, which express high levels of AR (Katz et al., 1989; Yu et al., 1993). It is possible that overexpression of AR results in changes in the levels of *ras, myc,* or other protooncogenes in C2 cells, which may in turn lead to posttranscriptional changes in Na channel proteins. Finally, in both neurons and muscle cells, the native Na channels comprise both an α-subunit and small molecular weight β-subunits (Barchi, 1983; Catterall, 1992). It has been suggested that assembly and insertion of neuronal Na channels in brain is increased by the association of the β-subunits with the α-subunit and by α-subunit glycosylation (Schmidt et al., 1985; Catterall, 1992). It is possible that overexpression of AR in C2 cells could led to a decrease in functional Na channel expression by decreasing expression of the β-subunit genes or by altering glycosylation of the α subunit. Future experiments addressing these potential mechanisms should provide significant insights into the molecular actions of steroid receptors, as well as the regulation of ion channel expression and function during differentiation of skeletal muscle.

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