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Activity regulates the levels of acetylcholine receptor α-subunit mRNA in cultured chicken myotubes

(allelic variants/restriction fragment-length polymorphism/control of mRNA accumulation/denervation hypersensitivity)

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ABSTRACT ***In vitro* blocking the spontaneous activity of primary cultures of chicken embryo myotubes with tetrodotoxin increases ≈2-fold their content in surface acetylcholine receptor. To investigate this effect at the level of gene expression, chicken genomic DNA sequences coding for the acetylcholine receptor α subunit were isolated and characterized. They were shown to belong to a single-copy, polymorphic gene with at least two alleles in the chicken strain utilized. Probes derived from these genomic clones were used to quantitate levels of α-subunit mRNA. In culture, a 2-day exposure to tetrodotoxin increased these mRNA levels up to 13-fold, a value similar to that observed after denervation of chick leg muscle (≈17-fold). Actin mRNA levels varied little in any of these experiments. These results support the notion that membrane electrical activity affects acetylcholine receptor expression by regulating the accumulation of the corresponding mRNAs.**

The acetylcholine receptor (AcChoR) is an oligomeric protein composed of five transmembrane subunits with an $\alpha_2\beta\gamma\delta$ structure (reviewed in ref. 1). In adult innervated muscle, the AcChoR is almost exclusively localized in the postsynaptic membrane of the neuromuscular junction, where its density is ≈1000-fold higher than that outside the junction (2). Denervation results in an enhanced sensitivity to acetylcholine all along the muscle fibers (3) because of a rise in the extrajunctional density of AcChoR following an increase in its rate of synthesis (4). This in turn is related to an increase in specific mRNA content, as demonstrated in rat with an α-subunit cDNA probe (5). Direct electrical stimulation of the denervated muscle prevents or reverses denervation hypersensitivity (4, 6, 7), indicating that muscle activity plays a significant role in this regulation.

Similar results have been obtained *in vitro* with chicken embryo myotubes. Blocking their spontaneous contractions with tetrodotoxin (TTX) increases AcChoR content through *de novo* synthesis, whereas direct electrical stimulation has the opposite effect (8, 9). This system has been used in several attempts to identify the regulatory signals involved (9–11). Ca^{2+} ions and/or cyclic nucleotides appear as plausible candidates [although conflicting interpretations remain (10, 11)], but their exact roles have still to be determined. Moreover, many opportunities for post-translational control exist, in view of the complex sequence of events involved in the assembly, maturation, and transport of the oligomeric AcChoR (12).

To investigate the regulation of AcChoR biosynthesis by activity at the level of gene expression and further to establish cultured chicken embryo myotubes as a model system for such study, we have isolated chicken DNA sequences from a genomic library (13) by cross-hybridization with a *Torpedo marmorata* α-subunit cDNA probe (14). All the positive clones derived from a single-copy, although polymorphic, gene encoding the AcChoR α subunit in chicken. Subclones were used to obtain probes for the quantitation of α-subunit mRNA in TTX-treated and untreated cultures in parallel with measurements of surface AcChoR content. We report here the effect of TTX on α-subunit mRNA levels, which is also compared with that observed *in vivo* after denervation of the corresponding muscle of 6-day-old chicks.

MATERIALS AND METHODS

Isolation and Characterization of Genomic Clones. A genomic library of chicken DNA (13) was kindly provided by J.-M. Jeltsch (Laboratoire de Biologie Moléculaire des Eukaryotes, Strasbourg). Recombinant phage plaques ($\approx 6 \times 10^5$), corresponding to four to six haploid genome equivalents, were screened with a *T. marmorata* AcChoR α-subunit cDNA probe (14). The probe, a *Pvu* II–*Bgl* II 835-base-pair (bp) fragment purified from clone pα-2 (14) by standard methods, corresponded to amino acids 37–314 of the mature α subunit. It was nick-translated (15) to a specific activity of $2–4 \times 10^8$ cpm/μg by using [α-^{32}P]dATP (800 Ci/mmol, Amersham; 1 Ci = 37 GBq). Three rounds of low-density replating led to the isolation of six independent genomic clones. Plate stocks were prepared from well-separated plaques, and phage DNA was extracted (16). It was subjected to single and double digestions with various restriction enzymes, and fragment sizes were determined after ethidium-bromide staining of agarose gels. Some digests were used for Southern blotting (17). The orientation of the chicken DNA inserts was initially obtained by *Eco*RI and *Hin*dIII partial digestions (18).

Myotube Cultures and Muscle Denervation. Myoblasts were obtained from the leg muscles of 10- or 11-day-old chicken embryos and cultured as described (9). ^{125}I-labeled α-bungarotoxin (New England Nuclear, 80–160 Ci/mmol) binding was used as a measure of surface AcChoR (9) and was averaged on duplicate 35-mm dishes. Nonspecific binding was determined in the presence of 10 μM decamethonium and was subtracted from all values (≈5% of total binding). TTX (final concentration, 0.5 μg/ml; Sigma) was added on day 6 in culture. Total protein content was measured in Triton X-100 extracts by the Coomassie blue technique (Bio-Rad). For denervation experiments, the sciatic nerve of 6-day-old chicks was transected on one side as described (19), the other side serving as control.

Phage M13 Subcloning, DNA Sequencing, and Single-Stranded Probes. Phage fragments which hybridized with the *Torpedo* probe were isolated from agarose gels and subcloned into the appropriate sites of phage M13 mp8/mp9 vectors (20). Dideoxy sequencing (21) was performed with [^{35}S]dATP

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Abbreviations: AcChoR, acetylcholine receptor; TTX, tetrodotoxin; bp, base pair(s); kb, kilobase(s).

(600 Ci/mmol, Amersham) as the label. M13 subclones also were used to obtain single-stranded uniformly ^{32}P-labeled probes (22) for RNA and DNA blotting analyses and nuclease S1 mapping.

Cellular RNA and DNA Extractions. Total RNA was prepared from pools of two to five 100-mm culture dishes by cell lysis in NaDodSO$_4$/acetate (pH 5) buffer, followed by two successive extractions with hot phenol and precipitation with ethanol. Yields varied between 25 and 100 μg of RNA per dish, depending on the initial seeding density and possibly on the serum batch used. Muscle RNA was extracted (23) from pools of two to four muscles, yielding ≈1 mg of total RNA per g of tissue. Genomic DNA was prepared (24) from the liver of 10-day-old chicks of the Warren strain (Centre Avicole de l'Île de France, Arpajon).

RNA and DNA Blotting Analyses. RNA samples were electrophoresed in 1.2% formaldehyde-agarose gels and transferred to either nitrocellulose (Schleicher & Schüll) or diazobenzyloxymethylated (25) nylon membrane (Biodyne A, Pall, France S.A.), by standard procedures. Hybridization was performed according to manuals of membrane suppliers, with an α-subunit single-stranded probe (22) or an actin nick-translated probe [plasmid pAL41, generously provided by S. Alonso, which contains an 1150-bp mouse cDNA insert derived from cytoskeletal β-actin mRNA (24; Margaret Buckingham, personal communication)]. Genomic DNA was treated as described (26). Final washes of the RNA and DNA blots were routinely done in 30 mM NaCl/3 mM sodium citrate/0.1% NaDodSO$_4$ at 45°C (low stringency). Autoradiograms of the hybridized blots were scanned with a densitometer using appropriate exposures to obtain calibration curves.

RESULTS

Characterization of Genomic Clones Coding for the α Subunit of Chicken AcChoR. Six independent recombinant phages (Fig. 1*a*) were isolated from a chicken genomic library (13) by cross-hybridization with a *T. marmorata* cDNA probe (14) encoding approximately two-thirds of the AcChoR mature α subunit. These phages appeared to harbor closely related inserts of chicken DNA, as judged from the restriction patterns obtained with several restriction enzymes and from blotting experiments with *Torpedo* probes [the relative positions of *Eco*RI and *Hin*dIII sites were established directly (18)]. Three of the inserts (in λαch20, λαch50, and λαch90) appeared to be identical in all of the experiments and might have derived from the amplification of a single initial recombinant phage. Altogether, the inserts spanned a region of genomic DNA that was ≈28 kilobases (kb) long (Fig. 1*a*). The approximate location of the region containing sequences homologous to the *Torpedo* probe is also shown, together with the 5′- to -3′ orientation of the sense strand of the DNA, initially deduced from blotting experiments.

To further characterize these clones, several restriction fragments that hybridized with the *Torpedo* probe were subcloned into appropriate sites of M13 mp8 and mp9 vectors (20) for sequence analysis (21). One of these fragments contained two stretches with a high homology to two successive exons of the human α-subunit gene, P2 and P3 (27), bordered by putative splice junctions (28). Exon lengths (146 and 45 bp) were identical to those of their human counterparts, with a slightly longer intron between them (124 bp vs. 111 bp). These lengths were confirmed in nuclease S1 mapping experiments (data not shown). Fig. 1*b* shows a part of this nucleotide sequence, which includes the smaller exon; both strands were sequenced, one strand being derived from clone λαch03 and the other, from clone λαch05. The deduced amino acid sequence is given above the exon DNA sequence. The corresponding data are also shown for man (27) and *T. marmorata* (14), where they differ from those of chicken. Chicken and man share the same amino acid sequence, which differs from that of *T. marmorata* at two positions. Moreover, the amino-terminal sequence of the gel-purified α subunit of

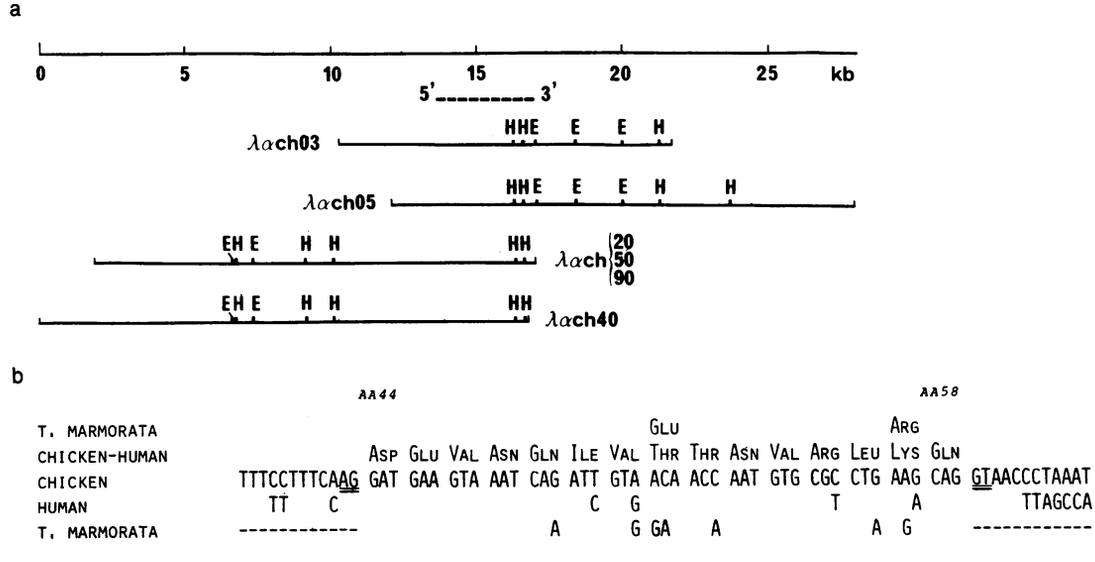

FIG. 1. (*a*) Diagrams of the chicken DNA inserts found in six independently isolated recombinant λ Charon 4A phages. These phages cross-hybridized with a *T. marmorata* probe coding for the AcChoR α subunit. Approximate location (obtained from blotting experiments) of sequences homologous to the probe is shown by the dashed line. The restriction map shows all *Eco*RI (E) and *Hin*dIII (H) sites within the inserts, except that sites for one enzyme clustered within less than ≈0.2 kb may have been represented as a single site. The 5′- to -3′ orientation of the gene was established by blotting experiments with various *Torpedo* probes and was confirmed by sequence analysis. (*b*) Nucleotide and amino acid (AA) sequences obtained from a region of chicken genomic clones encoding exon P3 (named according to ref. 27) of the AcChoR α subunit. The conserved dinucleotides (28) found at intron boundaries are underlined. Amino acid numbering is valid for all species studied so far and is defined starting at the amino-terminal residue of the mature subunit. The nucleotide differences found in the human (27) and *T. marmorata* (14) genes are displayed below the chicken sequence (no intron data is yet available for *Torpedo*). Chicken and man share the same amino acid sequence. The two residues that differ in *Torpedo* are indicated above the chicken sequence. Only one of the changes alters the amino acid type.

chicken muscle AcChoR (29) is identical to that deduced from exon P2 at 44 out of 47 positions (data not shown). This shows that all of our genomic clones derive from a chicken locus encoding the α subunit of the AcChoR.

A Single Polymorphic Locus Codes for the α Subunit of Chicken AcChoR. Several blotting experiments, such as that shown on Fig. 2*a*, indicated some degree of heterogeneity among the clones. Sequence analysis revealed that a base substitution was responsible for an additional *Pvu* II site in clone λαch05 and presumably in clone λαch40, thus splitting the 820-bp fragment A into fragments B and C. A few other base substitutions were also found (data not shown). In the region of exon P3, the sequences derived from fragments A and B were identical (Fig. 1*b*).

Thus, the possibility was considered that these genomic clones might have derived from two nonallelic genes. To resolve this point, genomic DNA was prepared (24) from the liver of 10 chickens of the Warren strain. A Southern blot of *Pvu* II digests of the 10 DNA samples was hybridized with ^{32}P-labeled fragment A subcloned from λαch03. All three fragments were present in half of the individuals tested (Fig. 2*b*, lanes 4–7), whereas in the other half, either fragment A alone (lanes 1 and 9) or fragments B and C (lanes 2, 8, and 10) were detected. A densitometric scan of such an autoradiogram showed that the intensity of a given band in the former group of individuals was on average half the intensity of the same band in the latter group.

The simplest interpretation of these results is that the chicken locus encoding the AcChoR α subunit is polymorphic. The proportions of heterozygotes (Fig. 2*b*, lanes 4–7) and homozygotes (Fig. 2*b*, lanes 1, 2, and 8–10) would be consistent with an ≈50% frequency for both alleles in the Warren chicken strain, although the existence of more than two allelic variants, differing at other sites, cannot be excluded. Moreover, this polymorphic gene is most likely a single-copy gene because no bands were observed other than those expected from the isolated genomic clones, even under low-stringency wash conditions (Fig. 2*b*); similar results were obtained with other restriction enzymes and another genomic fragment as probe (data not shown).

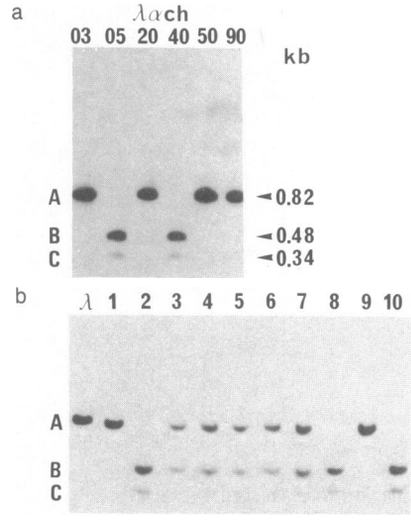

FIG. 2. Existence of two allelic forms of the AcChoR α-subunit gene in chicken, giving rise to a restriction fragment-length polymorphism. Southern blots of *Pvu* II digests of the six recombinant phages (*a*) and of the genomic DNA from 10 chickens (*b*) were prepared. They were hybridized with ^{32}P-labeled fragment A subcloned from genomic clone λαch03. After low stringency washing, the blots were autoradiographed for 24 hr. Fragment sizes (in kb) were determined by using phage λ–*Hin*dIII and phage φX174–*Hae* III digests as size standards. (*a*) Lane numbers refer to the numbering of the genomic clones (Fig. 1*a*). (*b*) Lanes: 1–10, genomic DNA (10 μg) prepared from the liver of 10 individual chickens; λ, fragment A (10 pg) purified from clone λαch03 and run together with the size standards.

Activity Regulates the Levels of AcChoR α-Subunit mRNA in Cultured Chicken Embryo Myotubes. After 5–6 days in culture, chicken embryo myotubes began to spontaneously contract, with only a variable minority of fibers actively contracting at any given time. Treatment with TTX (0.5 μg/ml), a blocker of the voltage-dependent Na^+ channel, resulted almost instantly in the complete cessation of contractions. No morphological changes were evident for the first 24–48 hr of TTX treatment. After 3 days, TTX-treated fibers were thinner than control untreated fibers and showed an ≈25% decrease in their total protein content (data not shown).

Surface AcChoR was quantitated by ^{125}I-labeled α-bungarotoxin binding (9) and normalized to the binding measured at the time of TTX addition. As shown in Fig. 3*a*, a 2-day exposure to TTX increased the number of surface AcChoR ≈2-fold relative to control untreated cultures. This relative increase was quite reproducible and consistent with previous studies (8–10). In one experiment, total AcChoR content was also measured on crude Triton X-100 extracts of the cultures and found to vary in proportion to surface AcChoR (data not shown).

RNA was extracted from parallel TTX-treated and control cultures with similar yields, and α-subunit mRNA was detected by blot-hybridizations of total RNA with a single-stranded probe containing the two exons described above

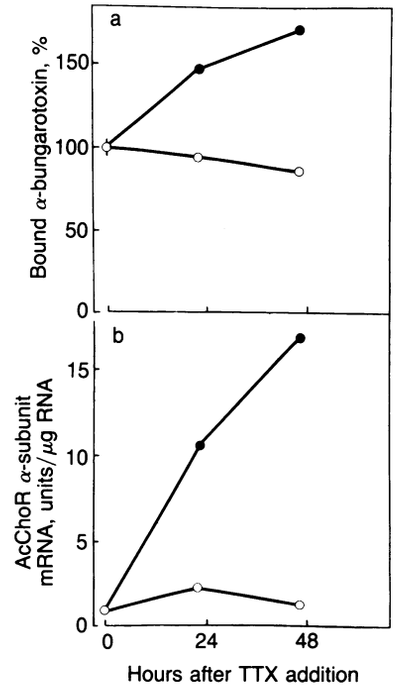

FIG. 3. Effect of TTX on surface ^{125}I-labeled α-bungarotoxin binding sites (*a*) and on AcChoR α-subunit mRNA (*b*) in cultured chicken embryo myotubes. On day 6 in culture, TTX (0.5 μg/ml) (●) was added to some dishes, the others serving as controls (○). (*a*) Toxin binding sites are expressed as a percentage of sites present at the time of TTX addition; they were usually in the range of 80–160 fmol per 35-mm dish. Differences between duplicate determinations appear as vertical bars but were generally smaller than the size of the symbols. (*b*) RNA was prepared from 100-mm dishes of the same culture batch. The amount of α-subunit mRNA is expressed as arbitrary units of film density per μg of total RNA, obtained by scanning autoradiograms of blot-hybridizations such as that in Fig. 4*a*.

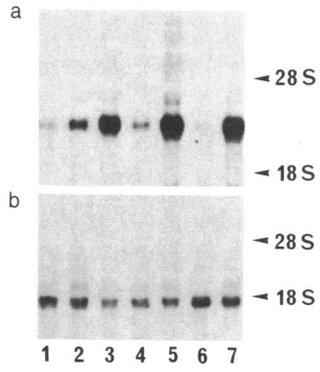

FIG. 4. Variations of α-subunit (*a*) and actin (*b*) mRNA levels in TTX-treated chicken embryo myotubes and in denervated chick muscle. Blots of total RNA were hybridized to a chicken AcChoR α-subunit probe (*a*) or to a mouse actin-specific probe (*b*) and were autoradiographed. Lanes: 1–5, RNA from cultured chicken embryo myotubes on day 0 (lane 1), day 1 (lanes 2 and 3), and day 2 (lanes 4 and 5) after TTX addition in control (lanes 2 and 4) or TTX-treated (lanes 3 and 5) dishes; 6 and 7, RNA from innervated (lane 6) and denervated (lane 7) chick muscle. RNA [10 μg per track in *a* and 6 μg in *b* except for lane 7 (10 μg)] was denatured with formaldehyde and formamide, electrophoresed in 1.2% formaldehyde-agarose gels, and transferred to a diazobenzyloxymethylated nylon membrane (*a*) or to nitrocellulose paper (*b*). The α-subunit probe was a single-stranded DNA fragment ≈820 nucleotides long containing two exons of this chicken gene (see text). The actin probe was plasmid pAL41, which contains a 1150-bp mouse cDNA insert derived from cytoskeletal β-actin mRNA, as determined from its 3′ untranslated sequence (24; Margaret Buckingham, personal communication). Some blots were hybridized with the second probe after the first was dehybridized, yielding results similar to those presented here. Size markers were chicken rRNAs.

(Fig. 4*a*, lanes 1–5). α-Subunit mRNA appeared as an ≈2.8-kb species in all RNA samples (minor bands were visible in overexposed lanes, such as lane 5). This is longer than the 2 kb found in *T. marmorata* (30) and mouse (31) but shorter than the 4.5 kb found in calf (27), probably reflecting variability in the length of the untranslated regions of these mRNAs. The same band was observed with poly(A^+) preparations (data not shown). Some blots were hybridized with a mouse actin-specific cDNA probe (Fig. 4*b*, lanes 1–5), identified as cytoskeletal β-actin from its 3′ untranslated sequence (24; Margaret Buckingham, personal communication). At the low stringency used, this probe hybridizes indifferently to the various actin mRNAs; the strongest band in Fig. 4*b* corresponds to sarcomeric α-actin (32).

TTX-treatment increased α-subunit mRNA levels relative to control, as estimated by densitometric scanning of autoradiograms, ≈4.5-fold 1 day after TTX addition and 13-fold after 2 days (Fig. 3*b*). In contrast, the levels of actin mRNA (Fig. 4*b*) were not significantly affected by the TTX treatment and decreased by ≈40% in both TTX-treated and control cultures over this 2-day period.

Denervation of Chick Leg Muscle Causes a Large Increase in AcChoR α-Subunit mRNA. Muscle denervation in rat has recently been shown to result in large increases in α-subunit mRNA levels (5). We found that this was also the case in chick muscle. RNA was extracted from denervated and contralateral innervated leg muscles of 10-day-old chicks, 4 days after transection of the sciatic nerve (19), and mRNA levels were determined as in the experiment described above (Fig. 4 *a* and *b*, lanes 6 and 7). Here again, α-subunit mRNA appeared as a single 2.8-kb species, levels of which were increased ≈17-fold by denervation (Fig. 4*a*). Under the same conditions, actin mRNA levels appeared to be somewhat decreased in denervated muscle (note the different amounts of RNA loaded in Fig. 4*b*, lanes 6 and 7). Interestingly, in this experiment, TTX-treated chicken embryo myotubes (Fig. 4*a*, lane 5) and denervated chick muscle (Fig. 4*a*, lane 7) contained similar amounts of α-subunit mRNA per μg of total RNA.

DISCUSSION

To investigate the regulation of the levels of AcChoR α-subunit mRNA in chicken embryo myotubes and chick muscle fibers, the genomic sequences encoding this mRNA were first characterized. Screening of a genomic library of chicken DNA (13) with a *T. marmorata* α-subunit cDNA probe (14) led to the isolation of six recombinant phages with overlapping inserts (Fig. 1*a*). They all were found to include two putative exons highly homologous to regions of the α-subunit gene in *Torpedo* (14, 33) and man (27) (Fig. 1*b*), consistent with the extensive sequence homology found between the nicotinic AcChoR from all vertebrate species studied so far. Exon lengths were the same as those proposed for the human gene (27). Their deduced amino acid sequence also agreed with the amino-terminal sequence of the gel-purified α subunit of chicken muscle AcChoR (29). Therefore, we are confident that these genomic clones derive from a chicken gene encoding the AcChoR α subunit.

Before using these clones to study the expression of the corresponding gene, we had to ensure that they harbored single-copy sequences. Southern blot experiments (Fig. 2*a*) had shown some degree of heterogeneity, confirmed by sequence analysis, among the six isolated clones. Low-stringency Southern blots of genomic DNA (Fig. 2*b*) revealed only the bands expected on the basis of the genomic clones (Fig. 2*a*), but the distribution of these bands among the 10 chickens tested showed the existence of two alleles for the chicken α-subunit gene (see below). The number of isolated clones (six in four to six genomic equivalents) is also consistent with a single-copy gene. We conclude that, within our detection limits, the α-subunit gene is encoded by a unique locus in chicken, as was found for *T. marmorata* (26) and mouse (31). Gene families could be suggested to account for the occurrence of α-bungarotoxin-binding sites and nicotinic AcChoR in the central nervous system of vertebrates (34), particularly in the optic lobe of the chicken (35), for developmental changes in several properties of the AcChoR (36), and for differences between the two agonist binding sites, carried mainly by the two α subunits present per pentameric AcChoR (1). All of the results obtained up to now indicate that, if such a gene family were to exist for the α subunit, then its members would not be very homologous to each other.

However, this single-copy gene is a polymorphic gene with a minimum of two alleles (Fig. 2*b*). Heterozygous individuals contain half the amount of each allele relative to the corresponding homozygotes, as expected. Both alleles appear to be equally distributed in the chicken strain used and in the genomic clones, within the limited statistics. The probable basis for this restriction fragment-length polymorphism is a single-base difference found in the analysis of two representative genomic clones (other single-base changes also were found). Such restriction fragment-length polymorphisms are not unusual in eukaryotic genomes and have proved to be useful in man and mouse for the chromosomal assignment of genes (37, 38). The linkage of this polymorphism with other markers also could be studied in chicken, although such genetic studies appear to be much less practical than in mouse.

Primary cultures of chicken myotubes are a well-established *in vitro* model to study the effects of muscle activity on AcChoR synthesis (9–11). Blocking the spontaneous activity of the myotubes with TTX caused an ≈2-fold increase in the number of surface ^{125}I-labeled α-bungarotoxin-

binding sites (Fig. 3*a*). This is a modest increase when compared with that observed *in vivo* after muscle denervation (5, 39). The levels of α-subunit mRNA were much more dramatically affected, increasing to 13-fold in our experiments (Fig. 3*b*), whereas actin mRNA levels changed little (Fig. 4*b*). The rapid kinetics of this effect (already a 4.5-fold increase after 24 hr) and its suppression by actinomycin D (4, 10) suggest that it reflects an enhanced transcription of the α-subunit gene.

Measurements of total AcChoR indicated that it was affected by TTX in the same way as was surface AcChoR. The discrepancy between the increases in α-subunit mRNA and surface ^{125}I-labeled α-bungarotoxin-binding sites thus cannot be accounted for by sequestration of newly synthesized AcChoR in a hidden pool (40). A similar discrepancy was observed in denervated rat muscle, where it could be explained by the shorter half-life of the newly synthesized, extrajunctional AcChoR (5). On the other hand, in a mouse clonal cell line, differentiation leads to a much larger increase in surface AcChoR than in α-subunit mRNA translatable *in vitro* (41). Further studies are necessary to elucidate the complex sequence of events linking the expression of the AcChoR subunit genes to the appearance of the mature oligomeric AcChoR on the cell surface (12).

To test further the relevance of our *in vitro* results, we also investigated the effects of *in vivo* denervation on chick leg muscle, the same muscle type as that from which the embryonic myoblasts used in the culture experiments were obtained. Denervation increased the levels of α-subunit mRNA ≈17-fold (Fig. 4*a*), again with little variation in actin mRNA content (Fig. 4*b*). Interestingly, TTX-treated myotubes and denervated muscle contained roughly similar amounts (taking into account the variability from one culture batch to the next) of α-subunit mRNA per μg of total RNA (Fig. 4*a*).

Other groups reported denervation-induced increases in α-subunit mRNA levels (5, 42). The quantitative differences between all observed increases could reflect either species or muscle-type differences. In fact, if the role of denervation is to "reactivate" (or derepress) the expression of AcChoR genes in extrajunctional nuclei (43), it can be argued that the increase in AcChoR mRNAs should be correlated with the ratio of extrajunctional to junctional nuclei in a given species and muscle type.

Our results thus show that the state of activity of *in vitro* differentiated myotubes specifically regulates the levels of mRNA coding for the α subunit of the AcChoR to an extent comparable with that observed after denervation of the corresponding muscle differentiated *in vivo*. Except for the probable involvement of Ca^{2+} ions and/or cyclic nucleotides (9–11), little is known about the signaling pathway underlying this regulation. One strong incentive for its further study comes from the attractive possibility that such mechanisms, linking the expression of specific proteins to membrane electrical activity, also may operate in nerve cells (44, 45). They could contribute at the molecular level to the engraving of external influences into long-lasting alterations of neuronal properties (1).

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