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Functional adult acetylcholine receptor develops independently of motor innervation in Sol 8 mouse muscle cell line

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We have defined culture conditions, using a feeder layer of cells from the embryonic mesenchymal cell line, 10T1/2 and a serum-free medium, which allow cells from the mouse myogenic cell line Sol 8 to form contracting myotubes for two weeks. Under these culture conditions, Sol 8 myotubes undergo a maturation process characterized by a sequential expression of two phenotypes. An early phenotype is typified by the expression of the nicotinic acetylcholine receptor (AChR) γ-subunit transcripts and the presence of low conductance ACh-activated channels, typical of embryonic AChR. A late phenotype is characterized by the expression of AChR ϵ-subunit transcripts, the decreased accumulation of γ-subunit transcripts and the appearance of high conductance ACh-activated channels, typical of adult AChR. These results indicate that the expression of functional adult type AChR does not require the presence of the motor nerve and therefore represents an intrinsic feature of the Sol 8 muscle cells. Chronic exposure of the cells to the voltage-sensitive Na^+ channel blocking agent tetrodotoxin does not affect the appearance of the AChR ϵ-subunit transcripts but prevents the reduction of the steady-state level of the AChR γ-subunit transcripts and yields a reduced proportion of the adult type channels. Thus, activity seems to facilitate the switch from the embryonic to the adult phenotype of the AChR protein. The Sol 8 cell system might be useful to analyse further the genetic and epigenetic regulation of muscle fibre maturation in mammals.

Key words: acetylcholine receptor transcript/patch-clamp technique/receptor channels/Sol 8 myogenic cell line

Introduction

Several functional properties of the nicotinic acetylcholine receptor (AChR) change during differentiation and maturation of skeletal muscle (for review, see Schuetze and Role, 1987). For instance, early noise analysis and synaptic current studies in amphibian and mammalian muscles have suggested that developmental modifications of AChR function can be assigned, at least in part, to changes in the ratio of two distinct channel types differing in their mean channel open time (Katz and Miledi, 1972, 1973). More recently single-channel studies in *Xenopus*, rat and mouse muscles have demonstrated that AChR channels are converted from an 'embryonic' form with low elementary conductance (35-40 pS) and long mean open duration to a mature 'adult' form, displaying high conductance (50-60 pS) and brief mean open duration (for review, see Brehm and Henderson, 1988).

Recombinant DNA technology has brought new insight into the nature of the developmental switch from embryonic to adult AChR channels. Expression studies of calf AChR mRNAs injected into *Xenopus* oocytes indicated that the main developmental change in AChR channel properties results from the substitution of the ϵ-subunit for the γ-subunit into the receptor protein at late stages of development (Takai *et al.*, 1985; Mishina *et al.*, 1986). This notion is further supported by developmental studies performed at mRNA and protein levels in calf (Mishina *et al.*, 1986) and rat (Gu and Hall, 1988; Witzemann *et al.*, 1989; Brenner *et al.*, 1990).

Although it is widely accepted that neural factors and electrical activity regulate AChR gene expression (for review, see Laufer and Changeux, 1989; Changeux *et al.*, 1990), their respective roles in promoting the conversion from the embryonic to the adult forms of the AChR are still far from clear (Brenner *et al.*, 1987; Brehm and Henderson, 1988). *In vivo* studies in rats have suggested that the conversion of embryonic to adult AChR results from a 'priming influence' from the motor nerve (Brenner, 1988; Gu and Hall, 1988; Brenner *et al.*, 1990). On the other hand, aneural *Xenopus* myotomal muscle maintained in culture expresses the same percentage of high conductance channels as *in vivo*, suggesting that conversion from embryonic to adult type of AChR is at best only weakly dependent on innervation, at least in this species (Brehm *et al.*, 1984; Owens and Kullberg, 1989a,b). Yet, aneural cultures of rodent muscle cells [rat primary cultures (Siegelbaum *et al.*, 1984) and mouse muscle cell line C2 (Changeux *et al.*, 1986), Sol 8 (Mulle *et al.*, 1988) and BC3H1 (Sine and Steinbach, 1986)] exhibit a very low level, if any, of adult type channels. The frequent instability of the myotubes formed *in vitro* rendered these cell systems poorly adapted to studies of muscle fibre maturation. We have thus defined culture conditions which allow cells from the mouse myogenic cell line Sol 8 to form stable spontaneously contracting myotubes. Under these conditions, we have obtained molecular and electrophysiological evidence for the appearance of the adult form of the AChR in the absence of motor nerve and muscle electrical activity.

Results

Culture of Sol 8 myotubes on feeder layer

In an attempt to improve the stability of the myotubes formed *in vitro*, we defined culture conditions which allow the maturation of Sol 8 cells over several weeks (see Materials and methods). They include the plating of Sol 8 myoblasts on a feeder layer of cells from the embryonic mesenchymal cell line, 10T1/2, treatment with mitomycin C and the

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induction of differentiation of myotubes in serum-free medium. Under these conditions, fusion occurred two days after plating and spontaneous contractions of myotubes were observed from day 6 and often persisted for two weeks. The time course of fusion of Sol 8 myoblasts directly plated onto plastic dishes was similar but contractions were rare. Furthermore, myotubes differentiated directly on plastic dishes progressively lost their elongated morphology and eventually detached (Figure 1a). In contrast, when plated on a feeder layer, Sol 8 cells formed elongated myotubes, stable for >2 weeks (Figure 1b,c). Ultrastructural studies (Figure 2) revealed that these myotubes, unlike cells grown on plastic, contain multiple, well organized sarcomeres. We thus analysed the expression and the functional properties of AChR from Sol 8 muscle cells cultured on a feeder layer.

Surface AChR and mRNA expression

The level of surface AChR (Figure 3), as assayed by iodinated α-bungarotoxin, increased steeply from day 2 and reached a plateau at day 4. During the following days, the number of α-bungarotoxin binding sites and the protein content per dish remained approximately constant.

Expression of α-, γ- and ϵ-subunits was studied at the RNA level. Northern blot analyses (Figure 4) and their quantification (Figure 5) show time courses of induction and accumulation of α- and γ-subunit mRNAs. A low level of α-subunit transcript could be detected in unfused cells. Accumulation of this transcript increased until reaching a plateau at day 5. The γ-subunit transcript was not detected in unfused cells. The steady-state level of this transcript then increased from day 3 to reach a peak value between day 6 and day 8. The level of accumulation of this transcript then progressively decreased. The quantification of the γ-subunit transcript between day 10 and day 12 represented $32 \pm 13\%$ of the maximal value ($100 \pm 20\%$) obtained between day 5 and day 8.

The presence of the ϵ-subunit transcript was not detected by RNA blot analysis and was assayed by the more sensitive procedure of cDNA-PCR (see Materials and methods). The ϵ-subunit transcript could not be titrated until day 3 (Figures 4 and 5). A low level of transcript was first detected at day 5, at which time maximal values had already been reached for α- and γ-subunits. Accumulation of the ϵ-subunit transcript then increased to reach a plateau around day 8. A similar delay in the appearance of the ϵ-subunit transcript was observed for cells differentiating in the absence of feeder layer, on plastic dishes (Figure 4). Since cells differentiated on plastic dishes frequently detached from dishes after eight days, this process was difficult to analyse at later stages in culture. Expression of the ϵ-subunit transcript was also detected in myotubes from the mouse myogenic cell line C2 (data not shown).

In order to estimate the relative amount of the γ- and ϵ-subunit transcripts we also analysed the level of the γ-subunit transcripts by the cDNA-PCR procedure. On day 8, the level of the γ-subunit transcripts was 40-fold higher than the level of the ϵ-subunit transcripts and then decreased to become only 15-fold higher at day 12 (see Materials and methods and Figure 8). These results thus establish that cloned mouse muscle cells express AChR ϵ-subunit transcripts in the absence of motor innervation but with a significant delay compared to the α- and γ-subunit transcripts.

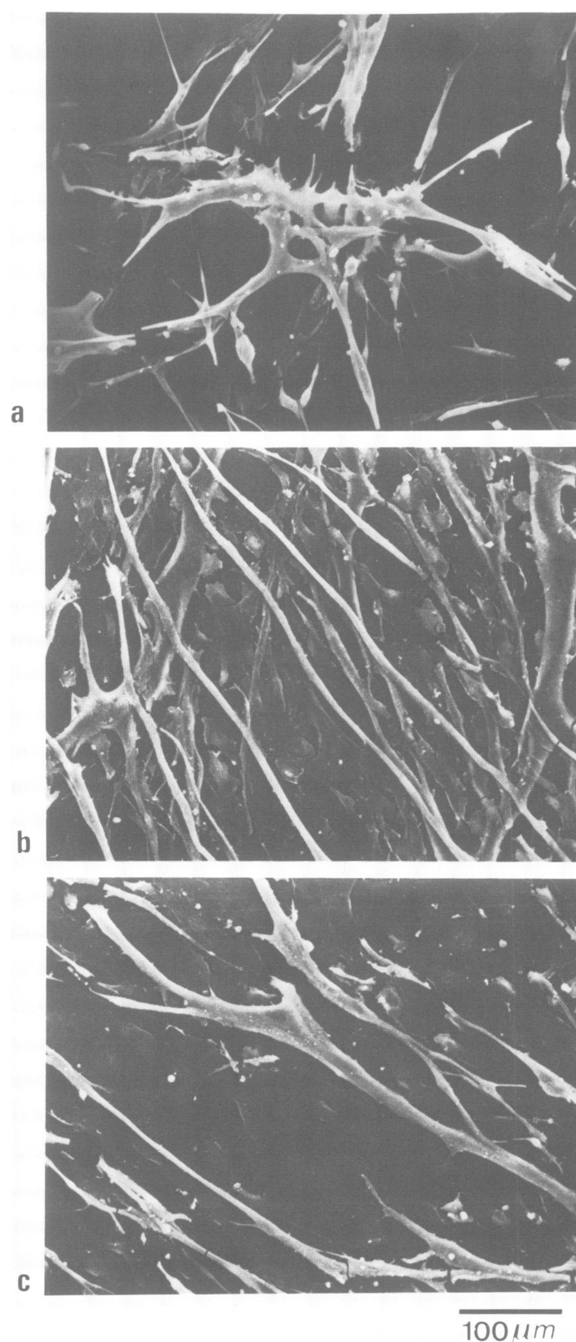

Fig. 1. Scanning electron micrographs of Sol 8 cells plated on plastic (**a**) and feeder layer (**b**,**c**) and observed 8 days (**a**,**b**) and 16 days (**c**) after plating.

Functional properties of AChR channels in myotubes cultured on a feeder layer

The functional properties of ACh-activated channels during this *in vitro* maturation process were studied by patch clamp techniques. The frequency of ACh-activated channel openings rose rapidly during the first 2–3 days in culture and afterwards no striking changes in opening frequency were observed. Channel activity was found at almost 100% of the recording sites after two days in culture. During the first 3–4 days, at almost all recording sites, we only detected low conductance channels (Figure 6A) with a slope conductance of 32.7 ± 0.9 pS ($n = 7$) (Figure 7A,B) typical

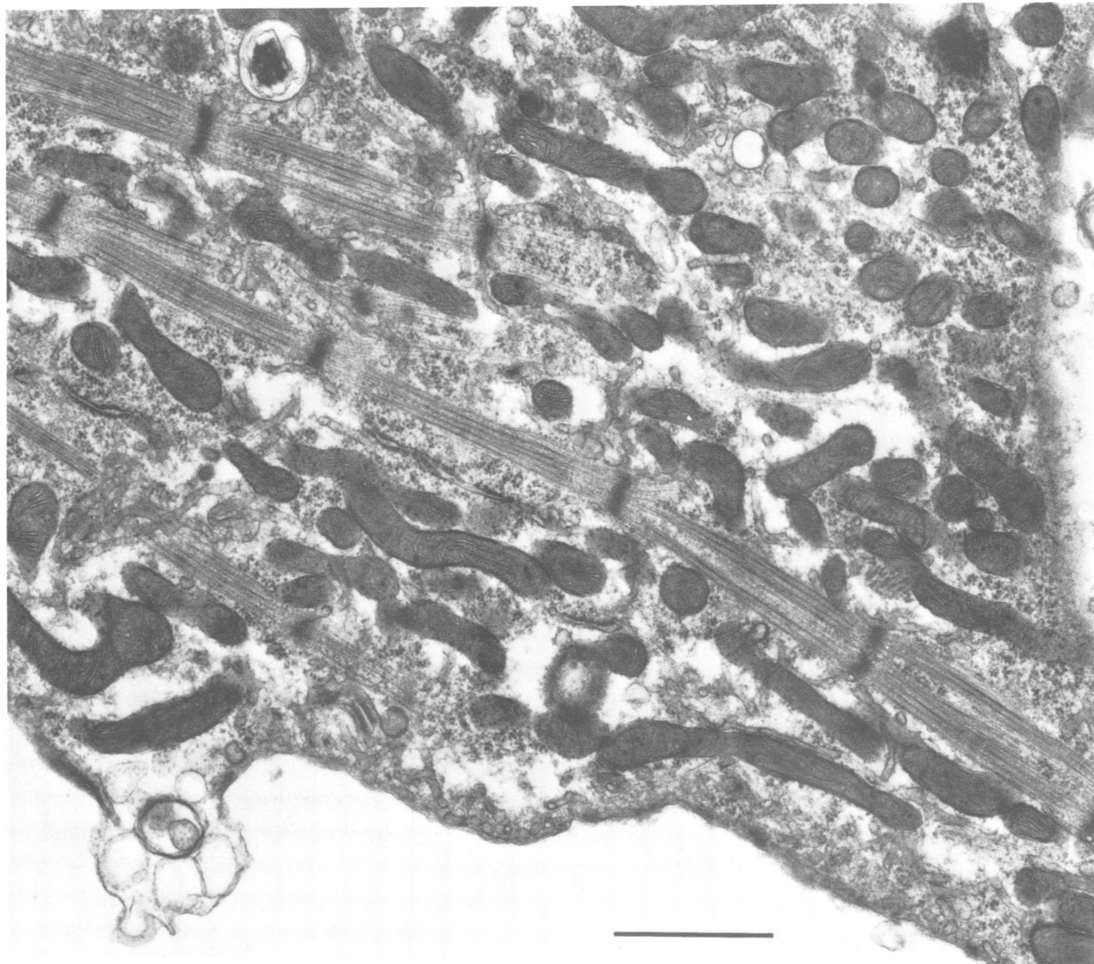

Fig. 2. Ultrastructure of Sol 8 cells grown for 19 days on feeder layer. Bar: 1 μm.

of the embryonic type of AChR channels (for review, see Brehm and Henderson, 1988). The distribution of open times for the low conductance channel could be fitted by a double exponential function (Figure 7C) as previously observed when low concentrations of agonist were used (Labarca *et al.*, 1985). The fast component was of the order of 0.5 ms (it may correspond to the opening of single liganded AChR). The value of the slow component appeared quite variable; for instance, in one experiment the values ranged between 3 and 13 ms, with a mean value of 6.5 ± 2.2 ms (n = 148 patches). As the myotubes developed in culture, a new population of ACh-activated channels with high conductance appeared (Figure 6B). These high conductance channels had a slope conductance of 47.4 ± 1.1 pS (n = 7) (Figure 7A,B) characteristic of the adult type of AChR. Open duration histograms for the high conductance channels were fitted by a single exponential function (Figure 7D) with an average time constant ranging between 1 and 2 ms with a mean value of 1.53 ± 0.37 (n = 20).

We measured the fraction of high conductance channel openings every 2–3 days for as long as two weeks in culture at 10–20 distinct recording sites for each day. The number of patches displaying high conductance channel openings increased steeply after three days in culture. Patches with high conductance channels reached a proportion of >90% after 8–10 days in culture. The relative frequency of high

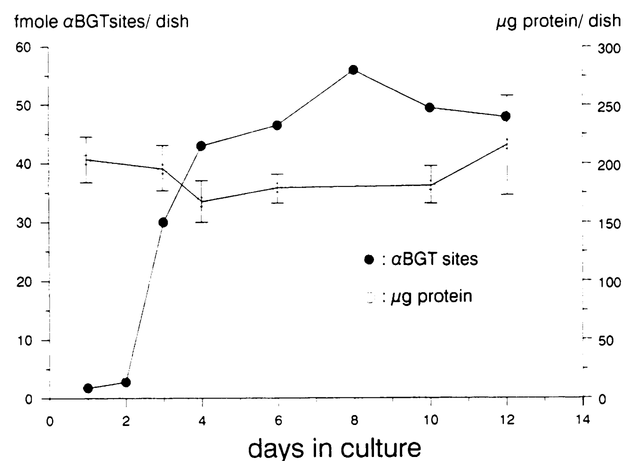

Fig. 3. Kinetics of accumulation of AChR and total proteins in Sol 8 cells grown on feeder layer. Surface AChR (●---●) was determined by binding of [^{125}I]α-bungarotoxin (see Materials and methods). Results are expressed as fmol of AChR per 50 mm dish and represent the mean of duplicate determinations. Amounts of total proteins (○---○) were measured using the BioRad assay. Results are expressed as mg of protein per 50 mm dish and correspond to the mean of triplicate determinations ± SEM.

conductance channels at individual recording sites also increased steeply after 7–8 days in culture as illustrated in Figure 6C for a representative experiment. In this

experiment, the proportion of high conductance channels reached a mean value of 22% at day 14 (range 0–68%). As a first approximation, the proportion of high conductance channel openings appeared not to depend on a particular site on the myotube (for instance, central part or extremities). Because short events (≤ 0.4 ms) were ignored and because of possible differences in agonist affinity and desensitization properties, our estimates of relative event frequency may not represent the precise relative number of each channel type. Nevertheless, these results support the general conclusion that in this culture system the AChR undergoes a maturation process that leads to the functional expression of high conductance adult type channels.

The relative proportion of high conductance channels formed by cells grown on plastic dishes was also examined. It must be noted that for cells grown on plastic dishes, the fraction of high conductance channels increased slightly with time but the relative proportion of high conductance channels only reached $0.8 \pm 0.6\%$ ($n = 4$) after 11–15 days in culture as compared to $14.5 \pm 4.3\%$ ($n = 7$) for cells grown on feeder layer. We tested whether the positive influence of the feeder layer could be exerted by purified attachment factor (collagen, fibronectin, laminin) or matrix obtained after the feeder layer was lysed with NaOH or fixed with ethanol. None of these culture conditions led to the appearance of a proportion of high conductance channels larger than the one found when cells were grown directly on plastic (data not shown).

Role of electrical activity in maturation of AChR

Cells grown on a fibroblast layer spontaneously contracted starting from around day 6. Tetrodotoxin (TTX) (10 μM), a blocker of the voltage-sensitive Na^+ channel, completely abolished these contractions (Weiss and Horn, 1986). Chronic exposure to TTX did not affect the formation of myotubes and did not significantly modify the time course of appearance and evolution of surface AChR as assayed by iodinated α-bungarotoxin binding (data not shown). Similarly, the time course for accumulation of α- and ϵ-subunit transcripts was not modified by the presence of TTX (Figure 8). In contrast, chronic treatment by TTX did prevent the decrease in the accumulation of the γ-subunit transcript (Figure 8) which occurred under control conditions (Figures 4 and 5). The results of two independent experiments indicated that the average steady-state level of γ-subunit transcript expressed as a percentage of day 8 level was, at day 11, 103.5 in the presence of TTX compared to 36.5 in control samples. Northern blot and cDNA-PCR gave similar results (see Figure 8, γ1 and γ2 lanes). Furthermore, simultaneous cDNA-PCR analyses of γ- and ϵ-subunit transcripts allowed us to estimate the relative abundance of each transcript (see Materials and methods and legend to Figure 8 for details). The ratio between ϵ- and γ-subunit transcripts was of the order of 1:40 at day 8 and of the order of 1:15 at day 11. In the presence of TTX this ratio remained unchanged at days 8 and 11.

We then investigated whether, under the same conditions, chronic exposure to TTX could regulate the appearance of high conductance channels. High conductance channels were present despite a blockade of contractile activity by TTX. However, as illustrated in Table I, in three separate experiments the fraction of adult type channels was 24–75% lower than control after chronic exposure to TTX. Furthermore, we did not observe any increase in the overall frequency of openings of ACh-activated channels in the presence of TTX.

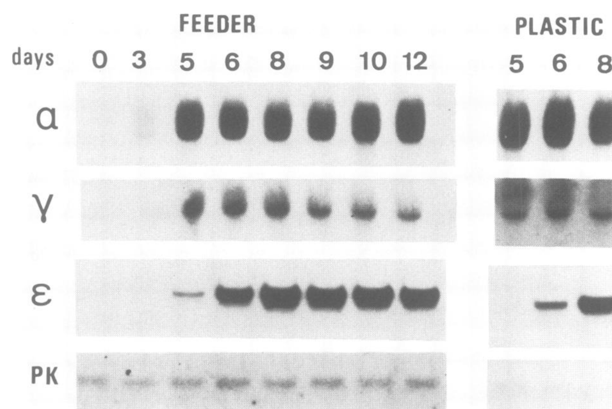

Fig. 4. Expression of AChR subunit transcripts during differentiation of Sol 8 cells grown on feeder layer (**a**) and plastic (**b**). α- and γ-subunit transcripts (RNA blot analysis), ϵ-subunit transcript (cDNA-PCR analysis). PK (pyruvate kinase) internal control of cDNA-PCR analysis.

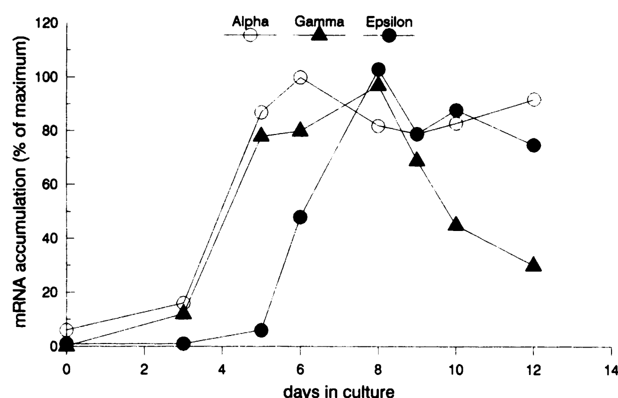

Fig. 5. Quantification after densitometry and integration of the results presented in Figure 4. Results corresponding to cells grown on feeder layer are expressed as a percentage of the maximum value determined at day 8 for γ- and ϵ-subunit transcripts and at day 6 for α-subunit transcript. (α, —○—), (γ, —▲—), (ϵ, —●—).

Discussion

We have studied the expression of transcripts for α-, γ- and ϵ-AChR subunits in parallel with electrophysiological properties of ACh-activated channels in the muscle cell line Sol 8 growing on a feeder layer of 10 T 1/2 cells. Under these culture conditions, Sol 8 myotubes undergo a maturation process which leads to the formation of stable, actively contracting myotubes with organized sarcomeres. Furthermore, this maturation process involves the sequential expression of two distinct phenotypes. An early phenotype is characterized by the expression of the γ-subunit transcripts and the presence of low conductance channels, characteristic of the embryonic AChR, while a late phenotype is typified by the expression of ϵ-subunit transcripts, the decreased accumulation of the γ-subunit transcripts and the appearance of high conductance channels characteristic of adult AChR. This sequence of events is reminiscent of *in vivo* development

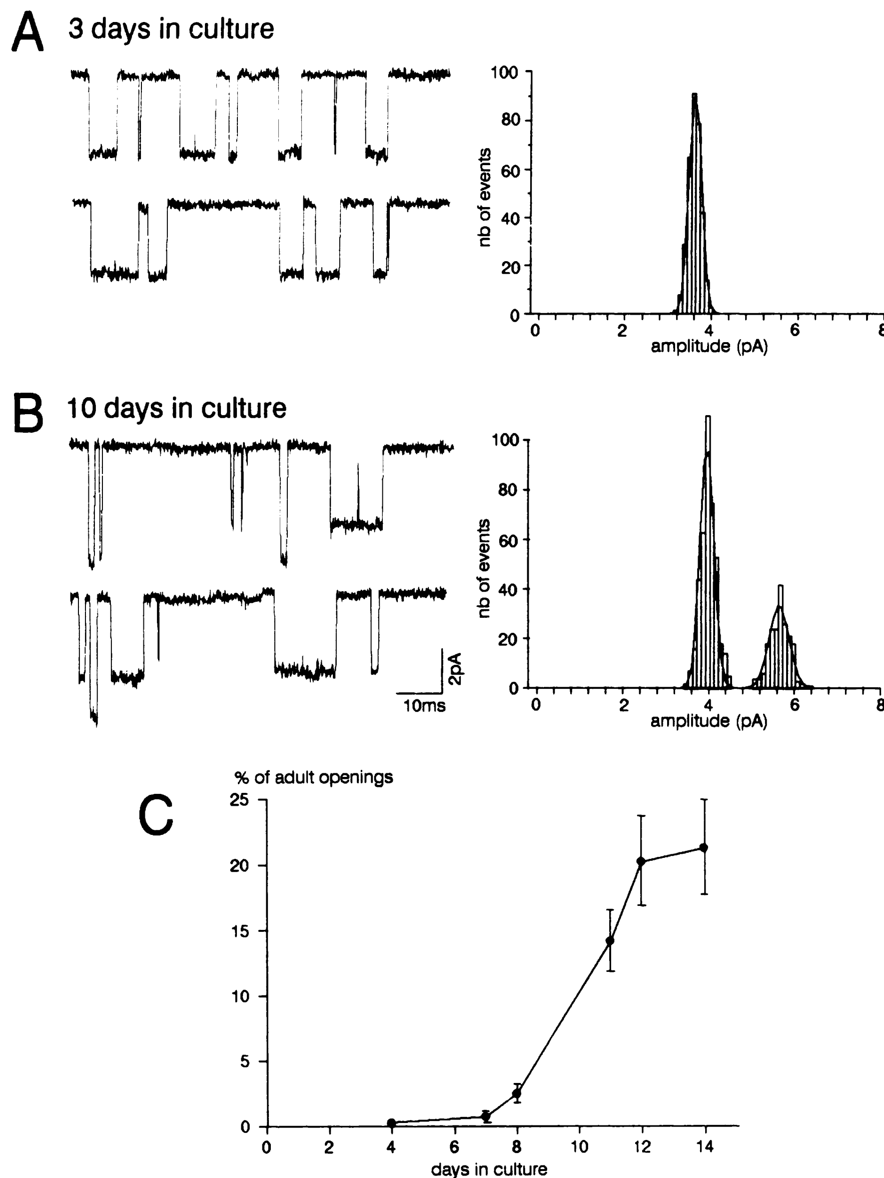

Fig. 6. **A** and **B**, single ACh-activated channels recorded in the cell-attached configuration on Sol 8 myotubes grown on a feeder layer for 3 days (A) and 10 days (B). Pipette potential was held at +50 mV. The patch pipette contained 500 nM ACh. On the right are given amplitude histograms showing a single population of channels at 3 days in culture and two distinct populations at 10 days in culture. **C**, relative frequency of high conductance versus total ACh-activated channels as a function of time in culture. Each point represents the mean (±SEM) of at least 10 separate measurements made at distinct recording sites.

(for review, see Brehm and Henderson, 1988; Witzeman *et al.*, 1989; Brenner *et al.*, 1990).

Only the early phenotype had previously been described, both by molecular and physiological methods, in cultured rodent muscle cells (Siegelbaum *et al.*, 1984; Buonanno and Merlie, 1986; Changeux *et al.*, 1986; Sine and Steinbach, 1986; Yu *et al.*, 1986; Evans *et al.*, 1987; Mulle *et al.*, 1988). However, recently, expression of the ϵ-subunit gene was detected in cultured mouse muscle cells (Martinou an Merlie, 1991). We show that when grown on a feeder layer, Sol 8 cells not only express the ϵ-subunit transcript but also a functional adult AChR. This indicates, as observed in *Xenopus*, that these late events do not require the presence of the motor nerve and therefore represent intrinsic features of the Sol 8 muscle cell. Our results thus contrast with the conclusion of Brenner *et al.* (1990) where the initial expression of the ϵ-transcript occurs *in vivo* only if a 'priming influence' is exerted by the motor nerve. At this stage, we cannot exclude the possibility that Sol 8 cells, which were initially isolated from satellite cells of young adult muscle, have kept a 'memory' of an early motor nerve influence upon the muscle from which they were derived. Yet, this possibility appears rather unlikely since myogenic derivatives of the embryonic mesenchymal cells, C3H 10 T 1/2, also express the ϵ-subunit transcript (D.Montarras and C.Pinset, unpublished observation; Martinou and Merlie, 1991).

Expression of the ϵ-subunit transcript occurs in Sol 8 myotubes, formed in the absence or presence of a feeder layer. However, myotubes formed in the absence of feeder layer systematically displayed a low percentage of high conductance channels. Thus, expression of the ϵ-subunit transcript did not suffice for the appearance of a high proportion of high conductance channels. Our attempts to replace the feeder layer by purified attachment factors

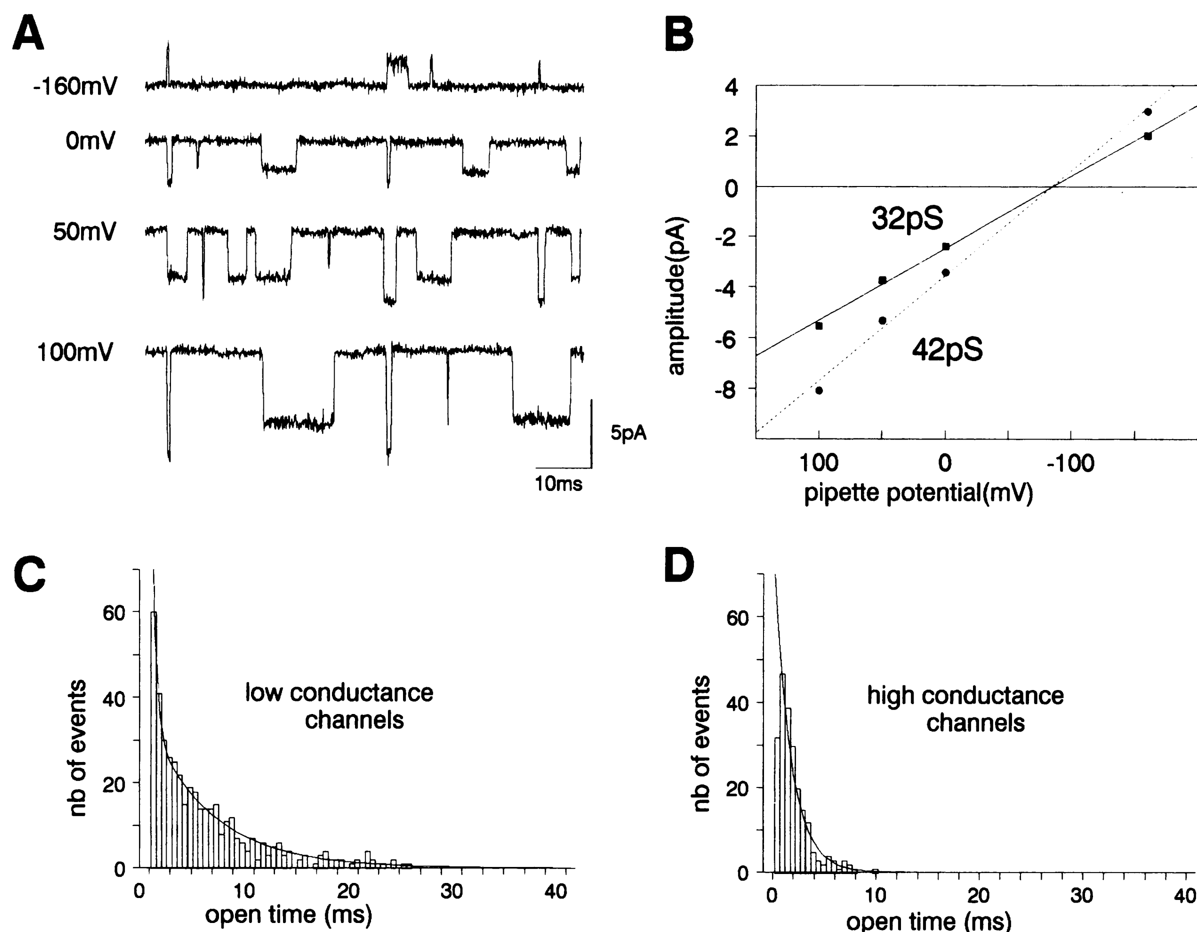

Fig. 7. Characteristics of ACh-activated channels recorded in Sol 8 cells grown on feeder for 10 days. Single channel amplitudes were measured at various pipette potentials (**A**) in order to determine the slope conductance of low and high conductance channels (**B**). Open time distribution for both types of channels (pipette potential, +50 mV) were fitted respectively by two exponentials for low conductance channels (**C**) and a single exponential for high conductance channels (**D**) (see Materials and methods).

(collagen, fibronectin and laminin) or cell matrix did not result in the appearance of high conductance channels. This suggests that the development of functional adult AChR at the cell surface requires 'complex' matrix and/or intercellular interactions between feeder cells and muscle cells. The nature of these interactions remains to be elucidated.

During the development of avian and rodent neuromuscular junction, muscle electrical activity leads to repression of extrajunctional AChR (for review, see Laufer and Changeux; Changeux *et al.*, 1990). Muscle electrical activity has also been reported to regulate channel conversion (Brenner *et al.*, 1987; Brenner, 1988). In primary cultures of chick and rat myotubes which display spontaneous contractile activity, a down-regulation of surface AChR occurs (Cohen and Fischbach, 1973; Rubin, 1985; Osterlund *et al.*, 1989). In chick, such a decreased level of surface AChR is associated with a decreased accumulation of α-subunit transcripts (Klarsfeld and Changeux, 1985) while steady-state levels of γ- and δ-subunit transcripts remain unchanged (Osterlund *et al.*, 1989). To our knowledge, no such data are available for mouse muscle primary cultures or cell lines. In our mouse *in vitro* Sol 8 system, activity regulates neither the total number of surface AChR nor the accumulation of α-subunit transcript. Furthermore, the expression of the ϵ-subunit transcripts appears independent of muscle activity. In contrast, chronic exposure to TTX prevents the decrease in the accumulation of the γ-subunit

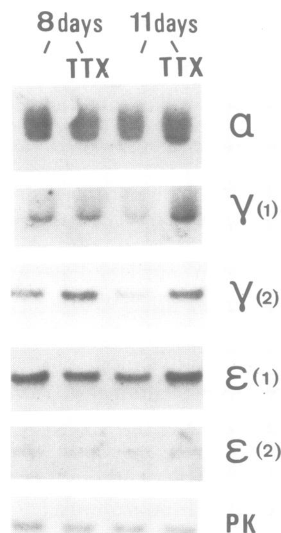

Fig. 8. Influence of TTX on accumulation of α-, γ- and ϵ-subunit transcripts. α-Subunit transcript: RNA blot analysis; γ-subunit transcript: RNA blot analysis $\gamma(1)$ and cDNA-PCR analysis $\gamma(2)$. In $\gamma(2)$, detection was performed using an oligonucleotide internal to the amplified fragment (see Materials and methods). ϵ-Subunit transcript: cDNA-PCR analysis. $\epsilon(1)$ detection was performed using the exon 10 labelled by random priming, and $\epsilon(2)$ detection was performed with the oligonucleotide complementary primer (see Materials and methods).

Table I. Influence of TTX on the relative frequency of high conductance ACh-activated channels

	Experiment 1	Experiment 2	Experiment 3
Control	17.8 ± 5.3% (n = 11)	6.8 ± 1.6% (n = 21)	14.8 ± 3.9% (n = 32)
TTX	12.8 ± 3.6% (n = 15)	1.8 ± 1.0% (n = 18)	7.4 ± 1.6% (n = 33)
	−24%	−75%**	−50%*

Mean values are indicated ± SEM. The decrease is significant at $^{**}P = 0.01$ and $^{*}P = 0.05$.

transcripts and reduces the proportion of functional adult type AChR. Since ε-subunit mRNA level is not affected by muscle activity, the ratio between functional embryonic and adult types of AChR in this system should thus be controlled by the relative abundance of the γ-subunit mRNA. Indeed, while at day 12 the ratio between ε- and γ-subunit mRNAs is of the order of 1:15, the ratio between adult and embryonic channels is of the order of 1:6. Considering the variability inherent in the biological system and in the techniques of detection, these data are consistent with the notion that the relative abundance of each channel type is, at least in part, regulated at the transcription level.

Our results do not imply that the establishment of the adult phenotype as it occurs *in vivo* (complete replacement of the embryonic AChR by the adult AChR) is independent of neural factors. However, they indicate that the maturation of AChR during muscle fibre development could be determined by two types of event. A gene expression programme, intrinsic to the muscle cell, would determine the sequential expression of γ- and ε-subunit mRNAs. In parallel, epigenetic events, such as muscle activity and neural factors which are responsible for the induction of synaptic AChR and repression of extrasynaptic AChR, would govern the expression of the γ-subunit gene. The system of Sol 8 cells grown on feeder layer should thus be useful to investigate further the mechanisms involved in the switch from embryonic to adult type AChR, as well as other aspects of muscle fibre differentiation and maturation.

Materials and methods

Cells and tissue culture products

MCDB 202 medium containing 50 mg/l of L-cysteine and DME medium were from Biochrome (Angoulême, France). Fetal calf serum (FCS) was from Jacques Boy (Reims, France).

10T1/2 cells (Reznikoff *et al.*, 1973) were maintained in a 1:1 mixture of MCDB 202 and DME supplemented with 2% Ultroser (steroid free) (IBF, France) and subcultured twice a week. Sol 8 myoblasts (Montarras *et al.*, 1989) were seeded at a density of 10^2 cells/cm^2 every 5 days in MCDB 202 medium supplemented with 20% FCS. All cultures were performed at 37°C under a humidifed atmosphere of air plus 7.5% CO_2.

To obtain feeder layers, 10^4 10T1/2 cells were seeded in 35 mm dishes. After 4 days, cells were treated with 2 μg/ml of mitomycin C (Sigma) for 4 h to irreversibly inhibit DNA replication. At this time, after extensive washings with medium, the feeder layers were ready to receive Sol 8 myoblasts. Two to three days prior to being plated on the feeder layer, Sol 8 myoblasts were grown in MCDB 202 medium supplemented with 20% FCS and 10^{-6} M dexamethasone (Sigma). Then cells were seeded on feeder layer or on plastic in the same medium, at a density of 4×10^4 cells/35 mm dish. This step defines the time zero of the experiments presented. One day later the medium was changed for a differentiation medium composed of MCDB 202 or DME supplemented with 0.2% Ultroser, 10 μg/ml bovine insulin (Sigma) and 10^{-6} M dexamethasone. To avoid medium changes, 3 ml of this medium were added to each dish.

Table II. Oligonucleotide primers

	Location in nucleotide sequence	Predicted size of amplified fragment
ε complementary primer (in exon 10)		
5′-CAGAATGCCCACAGACGAGGCACG-3′	3731−3708	
ε identical primer (in exon 9)		141 bp
5′-ACGCCAACGACTCATGCTACAT-3′	3518−3539	
γ complementary primer (in exon 10)		
5′-ATCCTGGACAGCAGCTGGAGCTAG-3′	1107−1084	
γ identical primer (in exon 9)		106 bp
5′-CCACACACACTCCATGGCCCGTG-3′	1001−1023	
Pyruvate kinase complementary primer		
5′-GGGTCAGTTGAGCCACACTCG-3′	617−594	
Pyruvate kinase identical primer		67 bp
5′-AAGCAACGTAGCAGCATGGAA-3′	0−21	

RNA

RNA preparation, Northern blot analysis and densitometry were performed as described previously (Pinset *et al.*, 1988).

Probes

The mouse AChR α-subunit probe was a cRNA synthesized from pGEM3 vector containing a 2.1 kb cDNA insert using the Sp6 polymerase in the presence of [α-^{32}P]UTP, 800 Ci/mmol (Amersham). The mouse AChR γ-subunit probe was synthesized from a 1.8 kb cDNA insert cloned into M13mp18 in the orientation giving the message strand in the virus. Primed vector was elongated in the presence of [α−^{32}P]dCTP and dTTP, 800 Ci/mmol (Amersham). An AChR γ-subunit oligonucleotide probe corresponding to a 3′ non-coding region of the AChR γ-subunit mRNA (Yu *et al.*, 1986) was also used. It was obtained by specific priming of the oligonucleotide (42mer) 5′-GGAGTCAGCTATGAGGGCCATGCT-GTTTGTAGAGCTGTATCC-3′ with the oligonucleotide (8mer) 5′-GGA TAC AG-3′. For cDNA-PCR analyses, the following probes were used: the mouse AChR ε-subunit probe was a DNA fragment corresponding to exon 10 of the ε-subunit gene (Buonanno *et al.*, 1989). This fragment was obtained by PCR from a genomic clone provided by J.P.Merlie. The amplified fragment was gel-purified and labelled by random priming (Feinberg and Vogelstein, 1984). The ε-subunit mRNA complementary primer was also used as a probe, when comparing the relative abundance of ε- and γ-subunit mRNAs. The γ-subunit probe was the oligonucleotide 5′-CCAGCTGTTACGGATGCATGTGCG-3′, internal to the amplified fragment. The use of divergent oligonucleotides and stringent amplification conditions ensured the specificity of the products amplified from the γ- and ε-subunit transcripts. The rat pyruvate kinase probe was the oligonucleotide 5′-CTTCGACGTGCGAGTCTGGCT-3′, internal to the amplified fragment. Oligonucleotides were labelled by kination in the presence of [γ-^{32}P]ATP, 3000 Ci/mmol (Amersham).

Synthesis and amplification of specific cDNAs

Global single-stranded cDNA was synthesized by random priming from 2 μg of total RNA in 40 μl of Taq DNA polymerase buffer containing 100 pmol of hexamers (BioLabs), 1.25 mM each of dNTP, 40 U of RNasin and 200 U of MMLV reverse transcriptase (BRL) at 42°C for 1 h.

Amplification was performed on one-quarter of the cDNA in 50 μl of Taq DNA polymerase buffer containing 100 pmol of the oligonucleotide primers complementary and identical to the transcripts (Table II) and 5 μl DMSO. After 10 min at 95°C, 2 U of Taq DNA polymerase were added. This step was followed by 15−30 cycles of amplification (denaturation for 30 s at 95°C; annealing for 30 s at 55°C; elongation for 1 min at 72°C). To prevent evaporation solutions were covered with mineral oil. As an internal control of amplification to compare the relative amounts of transcripts, 200 ng of total RNA from rat liver were added to each sample to perform co-reverse transcription and co-amplification of γ- and ε-cDNA with the rat liver pyruvate kinase cDNA (Chelly *et al.*, 1990). No cross-reactivity was detected between mouse myogenic cell RNA and rat pyruvate kinase oligonucleotides or between rat liver RNA and mouse γ- and ε-oligonucleotides. Analysis of the amplified products (gel electrophoresis, blotting and hybridization) was as described previously (Montarras *et al.*, 1989). Levels of amplification of the cDNA fragment were determined by densitometry and expressed relative to the level of amplification of the pyruvate kinase cDNA fragment that was used as a standard.

Scanning electron microscopy

After fixation in 2% glutaraldehyde cells were subjected to osmium tetroxide fixation in 1% OsO_4 and dehydrated in ethanol. Then cells were treated with hexamethyldisilazane for 5 min at room temperature and air dried. Metallization was performed by depositing gold palladium to a thickness of 4 mm. The microscope (Jeol JSM 350 F) was operated at 25 kV.

Transmission electron microscopy

Cells were fixed in 0.1 M Na^+ cacodylate, pH 7.2, 2.5% glutaraldehyde, 0.1 M sucrose, then post fixed in 0.1 M Na^+ cacodylate, 1% OsO_4, 0.1 M sucrose for 60 min at room temperature. Uranyl acetate staining was performed at room temperature in Michaelis buffer. Then cells were dehydrated in ethanol and embedded in Epon resin and viewed with a Zeiss EMlog transmission electron microscope.

Surface AChR determination

The number of surface AChR was determined by incubating living cells in the presence of 10 nM [^{125}I]bungarotoxin (sp. act. 200 Ci/mmol, Amersham) as described previously (Pinset *et al.*, 1988).

Electrophysiology

Standard single-channel recording techniques were used (Hamill *et al.*, 1981). All recordings were obtained from cell-attached patches from morphologically differentiated myotubes. Experiments were performed at room temperature. Before recording, the dish was bathed with standard extracellular solution containing 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES–NaOH and 10 mM D-glucose, pH 7.2. TTX (5 μM) was added just before recording in every case where the myotubes displayed spontaneous contractile activity. The patch pipette was filled with standard extracellular solution plus 500 nM ACh. This concentration of ACh elicited sufficient channel activity with minimal desensitization. It also minimized the occurrence of short openings of the low conductance channel, which have been ascribed to the opening of single liganded channels (Labarca *et al.*, 1985). Currents were measured with an Axopatch 1C (Axon Inst., Burlingame, CA) and were either stored on an FM tape recorder (Racal, Store 4) before analysis or directly digitized at 10 kHz. In both cases they were analysed with the Pclamp program (v. 5.03) of Axon Inst. (Burlingame, CA).

Pipette potential was set between +30 and +50 mV so that the amplitude of the low conductance ACh-activated channels was between 3.5 and 4 pA (assuming a conductance of ~35 pS, this gives a transmembrane potential of roughly −100 to −115 mV). Open durations and mean amplitudes of all classes of events were measured and compiled into histograms. All events shorter than 400 μs were ignored. Amplitude histograms were fitted by double Gaussian functions. At the membrane potential used, low and high conductance types of ACh-activated channels formed two clearly distinct populations of channels (the standard deviation for the Gaussian functions ranged between 0.1 and 0.3 pA, for mean amplitudes of ~4 and 6 pA respectively). The relative proportion of high conductance channels was thus estimated after construction of the histograms for all events longer than 400 μs. Open duration histograms were fitted by single- and double-exponential functions by the method of maximum likelihood separately for both populations of channels.

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