Activation of nicotinic acetylcholine receptors increases the rate of fusion of cultured human myoblasts

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1. Fusion of myogenic cells is important for muscle growth and repair. The aim of this study was to examine the possible involvement of nicotinic acetylcholine receptors (nAChR) in the fusion process of myoblasts derived from postnatal human satellite cells.

2. Acetylcholine-activated currents (ACh currents) were characterized in pure preparations of freshly isolated satellite cells, proliferating myoblasts, myoblasts triggered to fuse and myotubes, using whole-cell and single-channel voltage clamp recordings. Also, the effect of cholinergic agonists on myoblast fusion was tested.

3. No nAChR were observed in freshly isolated satellite cells. nAChR were first observed in proliferating myoblasts, but ACh current densities increased markedly only just before fusion. At that time most mononucleated myoblasts had ACh current densities similar to those of myotubes. ACh channels had similar properties at all stages of myoblast maturation.

4. The fraction of myoblasts that did not fuse under fusion-promoting conditions had no ACh current and thus resembled freshly isolated satellite cells.

5. The rate of myoblast fusion was increased by carbachol, an effect antagonized by α-bungarotoxin, curare and decamethonium, but not by atropine, indicating that nAChR were involved. Even though a prolonged exposure to carbachol led to desensitization, a residual ACh current persisted after several days of exposure to the nicotinic agonist.

6. Our observations suggest that nAChR play a role in myoblast fusion and that part of this role is mediated by the flow of ions through open ACh channels.

In vivo, muscle satellite cells are quiescent mononucleated cells lying on skeletal muscle fibres (Mauro, 1961; Campion, 1984). In response to various stimuli, these cells can proliferate as myoblasts and fuse, thereby contributing to growth, repair and hypertrophy of postnatal skeletal muscle. It has been shown in the rat that fibre-attached satellite cells do not express nicotinic acetylcholine receptors (nAChR) (Bader, Bertrand, Cooper & Mauro, 1988). However, proliferating myoblasts derived from satellite cells express nAChR (Cossu, Eusebi, Grassi & Wanke, 1987; Bader *et al.* 1988), suggesting that these receptors may be involved in the maturation of these cells. In embryonic rat myoblasts the possibility that nAChR were involved in the fusion process was examined and dismissed (Fambrough & Rash, 1971). In contrast, in chick embryonic myoblasts it has been found that nAChR activation modulates the fusion (Entwistle, Zalin, Warner & Bevan, 1988*b*). In view of the current interest in human satellite cells as therapeutic tools for treating genetic muscle diseases (myoblast transplantation) and general diseases (*ex vivo* somatic gene therapy) and given the importance of the fusion process in these applications, an evaluation of the role of nAChR in human myoblast fusion seems justified. An additional justification is that the pertinent results on this subject were obtained in embryonic myogenic cells. There are indications that results from these embryonic preparations may not always be directly applicable to postnatal myogenic cells, i.e. to satellite cells (Cossu & Molinaro, 1987).

The fact that acetylcholine (ACh) may be involved in the fusion process raises the question of its source during postnatal muscle growth or during muscle regeneration. There is evidence that the whole muscle itself may contain ACh in a compartment distinct from the nerve-ending compartment (Hebb, 1962; Miledi, Molenaar & Polak,

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1977; Miledi, Molenaar, Polak, Tas & Van der Laaken, 1982). It is possible that the muscle fibres contain and release ACh (Miledi *et al.* 1977), but this has never been demonstrated, as the whole muscle is made up of different cell types. In cultured chick embryonic myoblasts the presence of the ACh synthesizing enzyme choline acetyltransferase has been documented (Entwistle *et al.* 1988*b*). The same study showed evidence of ACh-like immunoreactivity. It was also suggested on the basis of indirect arguments that an ACh-like agent was released, but the source of this compound could not be ascertained as the cultures contained non-myogenic cells.

We have ways of preparing pure cultures of human satellite cells (Baroffio, Aubry, Kaelin, Krause, Hamann & Bader, 1993), and of recording from these cells and from their pure myogenic progenies (Bernheim, Krause, Baroffio, Hamann, Kaelin & Bader, 1993; Hamann *et al.* 1994). We therefore decided to examine in pure human myogenic cells the expression of nAChR, the presence of ACh and its synthesis and release, and the role of ACh and nAChR in the fusion process.

In the present paper we investigate the expression of ACh-activated currents (ACh currents) in cultured human satellite cells and in myoblasts and myotubes derived from these cells, and we also study the role of nAChR in fusion. Our results reveal that the expression of nAChR in myoblasts increases markedly before fusion and demonstrates that activation of nAChR increases the rate of myoblast fusion. In the companion paper (Hamann *et al.* 1995) we examine the synthesis and release of an ACh-like compound by pure human myogenic cells.

METHODS

Dissociation and culture procedures

Samples of healthy human skeletal muscle (0·36–2 g) were obtained during corrective orthopaedic surgery of eleven patients (aged between 3 and 19 years) in accordance with the guidelines of the ethical committee of the University Hospital in Geneva. Written informed consent was obtained from patients or their guardians.

The dissociation procedure used to isolate and prepare clonal cultures from satellite cells was described previously (Baroffio *et al.* 1993). Briefly, the muscle sample was cleaned, minced and incubated for 1 h at 37 °C in 0·05% trypsin. The cells were then centrifuged and resuspended several times in wash medium (F10 nutrient medium supplemented with 15% fetal calf serum (FCS)) in order to pellet muscular debris. Tris–ammonium chloride buffer was added to lyse red blood cells.

Single satellite cells were manually collected with a micropipette under a microscope and cultured individually in wells containing proliferation medium in which they actively divide (clonal culture). The proliferation medium was composed of F10 nutrient medium supplemented with 15% FCS, 0·5 mg ml^{-1} bovine serum albumin (BSA), 0·5 mg ml^{-1} fetuin, 10 ng ml^{-1} epidermal growth factor, 0·39 mg ml^{-1} dexamethasone, 0·18 mg ml^{-1} insulin and 0·1 μg ml^{-1} gentamicin (Ham, St Clair, Blau & Webster, 1989). When confluent, cells were replated at a lower density. Clonal cultures of myoblasts can proliferate for several months. Thirteen clonal cultures from nine biopsies were used in this study. Formation of myotubes can be induced by replacing the proliferation medium with a medium that promotes myoblast differentiation and fusion (St Clair, Meyer Demarest & Ham, 1992). The differentiation medium was composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 0·5 mg ml^{-1} BSA, 10 ng ml^{-1} epidermal growth factor, 10 μg ml^{-1} insulin and 1 μg ml^{-1} gentamicin. Half of the culture medium was changed 3 times a week.

The following terms will be used to describe the different stages of maturation of our preparation:

Freshly isolated satellite cells. Quiescent (non-dividing) mononuclear cells directly obtained from a muscle biopsy. Satellite cells were manually selected with a micropipette under a microscope and electrophysiological recordings were performed 20–28 h after collection when the cells were still non-dividing (Widmer, Hamann, Baroffio, Bijlenga & Bader, 1995). The properties of these cells should be very close to the properties of the *in vivo* quiescent satellite cells. Freshly isolated satellite cells from three biopsies were tested.

Proliferating myoblasts. Manually cloned satellite cells grown in the proliferation medium give rise to myoblasts that are actively dividing mononucleated cells. Clonal cultures were kept for periods of up to 100 days.

Myotubes. Multinucleated cells. Myotubes were obtained by exposing proliferating myoblasts reaching confluency (230 000 ± 10 000 cells per well; $n = 30$) to differentiation medium. Fusion of myoblasts to form myotubes began within 2 days. Myotubes used for electrophysiological recordings were kept in differentiation medium for 4–59 days.

Prefused myoblasts. Fusion-competent myoblasts prepared by seeding proliferating myoblasts in the differentiation medium for 4 days at very low density to avoid physical contact between cells (10 000 cells per well). In this condition, myoblasts should presumably be in a more mature state, but most of them are prevented from fusing due to the low cell density.

The relationship between the number of nuclei and the cell capacitance (directly given by the amplifier during whole-cell recording, see below) was studied in thirty prefused myoblasts (see inset of Fig. 3*A*). To minimize any cell damage that might have interfered with a precise determination of the number of nuclei, cells were patched only for the time necessary to measure the capacitance (i.e. approximately 30 s). Following capacitance determination, the cells were allowed to recover for 1 h in the incubator (during this time they flattened, which facilitated the counting of nuclei), fixed with 100% ethanol (30 s at room temperature, 20–22 °C), and stained with Haematoxylin to evaluate the number of nuclei. Based on the results of Fig. 3*A* inset, we chose 50 pF as the critical value to distinguish prefused myoblasts (mononucleated) from small myotubes (multinucleated).

Non-fusing myoblasts. This term refers to the myoblasts (20–30% of all myoblasts initially present before fusion) that do not fuse despite a prolonged culture in the differentiation medium. The recorded non-fusing myoblasts were kept up to 3 months in differentiation medium.

Table 1. Properties of ACh current and other characteristics of the various types of human myogenic cells studied

Cell type	Current density (pA pF^{-1})	Expression (%)	E_{ACh} (mV)	Desensitization (%)	γ (nS)	τ (ms)	Membrane capacity (pF)	Size (μm)	n (cells)
Satellite cells	0	0	—	—	—	—	5 ± 1	13	16
Proliferating myoblasts	6 ± 2*	54	0 ± 1 (4)	5 ± 4 (6)	43 (2)	7·0	24 ± 2	28	35
Prefused myoblasts	38 ± 13*†	93	2 ± 3 (4)‡	9 ± 3 (14)	42 ± 2 (5)	3·9	26 ± 3	29	15
Myotubes	49 ± 6	100	7 ± 2	3 ± 2 (5)	40 ± 2 (4)	4·0	171 ± 32	74	11
Non-fusing myoblasts	0	0	—	—	—	—	34 ± 0·2	33	14

Current density (pA pF^{-1}) was obtained during 500 ms exposure to 1 μM ACh. Expression, the percentage of cells expressing ACh current. E_{ACh}, the reversal potential of ACh current. Desensitization, the percentage desensitization measured at −80 mV during 500 ms ACh application (1 μM). γ, single-channel conductance. τ, single-channel mean open time at −80 mV calculated by fitting open time histograms. Size, cell diameter of an ideal spherical cell calculated from the membrane capacity (assuming a specific membrane capacitance of 1 μF cm^{-2}). n, number of cells tested. Numbers in parentheses refer to the number of cells used for the specific result. * Value calculated by assigning 0 pA pF^{-1} to cells without ACh current. † Significantly different from proliferating myoblasts ($P < 0·001$) but not from myotubes ($P = 0·51$). ‡ Not significantly different from myotubes ($P = 0·16$) and proliferating myoblasts ($P = 0·47$). E_{ACh} is marginally larger in myotubes than in proliferating myoblasts ($P = 0·04$), possibly reflecting a space-clamp problem (even though we recorded from small myotubes).

Electrophysiological recordings

Ionic currents were recorded under voltage clamp in either whole-cell or single-channel (outside-out) configuration of the patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) using an Axopatch-200A amplifier (Axon Instruments). Capacitances of the recorded cells were obtained from direct reading of the whole-cell capacitance potentiometer of the amplifier. Patch pipettes were made from borosilicate glass using a BB-CH puller (Mecanex, Switzerland) and coated with Sylgard (Dow Corning) when single-channel currents were studied. The resistance of the pipettes was between 2 and 5 MΩ. Currents were recorded at 20–22 °C, sampled at 1·7 kHz, and low-pass filtered at 1 kHz. To obtain spherical cells, which were more easily patch clamped, cells were treated with 0·05% trypsin and replated 20–24 h (myotubes) or 1–2 h (myoblasts) before recording. Note that treating myotubes again with trypsin a few minutes before recording did not significantly affect the ACh current densities.

Solutions and materials

The extracellular (control) solution was composed of (mM): 150 NaCl, 5 KCl, 5 $CaCl_2$, 2 $MgCl_2$, and 5 Hepes. The pH was adjusted to 7·3 with NaOH. The intracellular (pipette) solution was composed of either (mM): 150 KCl, 1 $MgCl_2$, 5 Hepes and 0·2 EGTA or 80 KF, 50 potassium acetate, 50 KOH, 1 $MgCl_2$, 10 Hepes and 10 BAPTA. Either of these two solutions were used for whole-cell recordings with no obvious differences in the results. For single-channel recordings, only the second solution was used. The pH of both intracellular solutions was adjusted to 7·2 with KOH.

Trypsin (from bovine pancreas) was from Boehringer-Mannheim. Ham's F-10 medium, DMEM and gentamicin were from Gibco. BSA, dexamethasone, fetuin, insulin, Hepes and decamethonium were from Sigma. Epidermal growth factor (EGF) was from Inotech (Dottikon, Switzerland). FCS was from Ready System (Zurzach, Switzerland) or Inotech. α-Bungarotoxin was from Calbiochem. NaCl, KCl, $MgCl_2$, $CaCl_2$, KOH and potassium acetate were from Merck. EGTA, BAPTA, tubocurarine chloride, carbamylcholine chloride (carbachol), atropine, acetylcholine chloride and KF were from Fluka (Buchs, Switzerland).

ACh application system

A computer-controlled application system was used throughout the experiments as described by Bernheim, Beech & Hille (1991). Briefly, a double-barrelled capillary tube (theta tube) was pulled to a diameter of around 200 μm and placed approximately 100 μm away from the cell. Each side of the theta tube was connected to an elevated reservoir. Reservoirs contained either control solution (see above) or control solution with 1 μM ACh. Both reservoirs were at the same level to avoid pressure-related artifacts when ACh was applied. Computer-controlled solenoid valves allowed us to switch the flow from one side of the theta tube to the other. A bath perfusion (about 3 ml min^{-1}) with control solution was constantly present during the use of the rapid application system. With the exception of the experiments in which the nAChR antagonist curare was tested (Fig. 2), the theta tube application system was used for all the electrophysiological experiments described in this study. While examining the effect of curare, ACh was pressure-applied using a pipette (100 μm diameter) placed approximately 100 μm from the myotubes or from the outside-out patches.

Fusion index

Cultures were fixed with 100% ethanol (30 s at room temperature, 20–22 °C) and stained with Haematoxylin. Nuclei were counted in ten randomly chosen microscope fields (2 culture dishes, 5 fields in each dish) at a magnification of ×400. One microscope field usually contained between 100 and 200 nuclei. The fusion index is defined as the number of nuclei in myotubes divided by the total number of nuclei.

Statistics

Results are expressed as means ± S.E.M.

RESULTS

The effect of ACh will be described first in freshly isolated purified satellite cells, then in cultures of proliferating myoblasts and in multinucleated myotubes. The purity of the cultures was ensured by seeding single satellite cells (clonal cultures).

Effect of ACh on freshly isolated satellite cells and non-fusing myoblasts

Previous studies in the rat indicated that satellite cells do not express ACh currents immediately after dissociation, when they are still attached to their muscle fibre (Bader *et al.* 1988). To test whether this was also true in human satellite cells, ACh (1 μM) was applied briefly on freshly isolated satellite cells (Fig. 1*C*) held at −80 mV. Figure 1*A* shows that no ACh current could be detected at this developmental stage, even when the currents were grossly magnified (note that the current scale is ±2 pA in this figure, as compared with the nanoamp scale in other figures). None of the sixteen cells tested had an ACh current. To exclude the possibility that the lack of ACh current could be a recording artifact due to poor patch perforation, voltage gated whole-cell currents were also tested. In all recorded cells, an outward potassium current (Hamann *et al.* 1994) could be elicited by a voltage step to +40 mV from a holding potential of −80 mV.

In cultures exposed to differentiation medium, most myoblasts fused to form myotubes. A few non-fusing myoblasts, however, were always present (Fig. 1*D*, arrowheads). We found that none of the non-fusing myoblasts examined had a measurable ACh current ($n = 14$). An example of the effects of ACh application on one of these cells is illustrated in Fig. 1*B*. Electrophysiologically, non-fusing myoblasts were distinguished from small myotubes on the basis of their membrane capacitance ($C \leqslant 50$ pF; see Methods).

Proliferating mononucleated myoblasts and multinucleated myotubes

In the appropriate culture medium, satellite cells give rise to myoblasts that proliferate actively. A photograph of Haematoxylin-stained proliferating myoblasts is shown in Fig. 2*E*. In these cells, ACh currents were small (see Table 1) and only observed in nineteen of the thirty-five

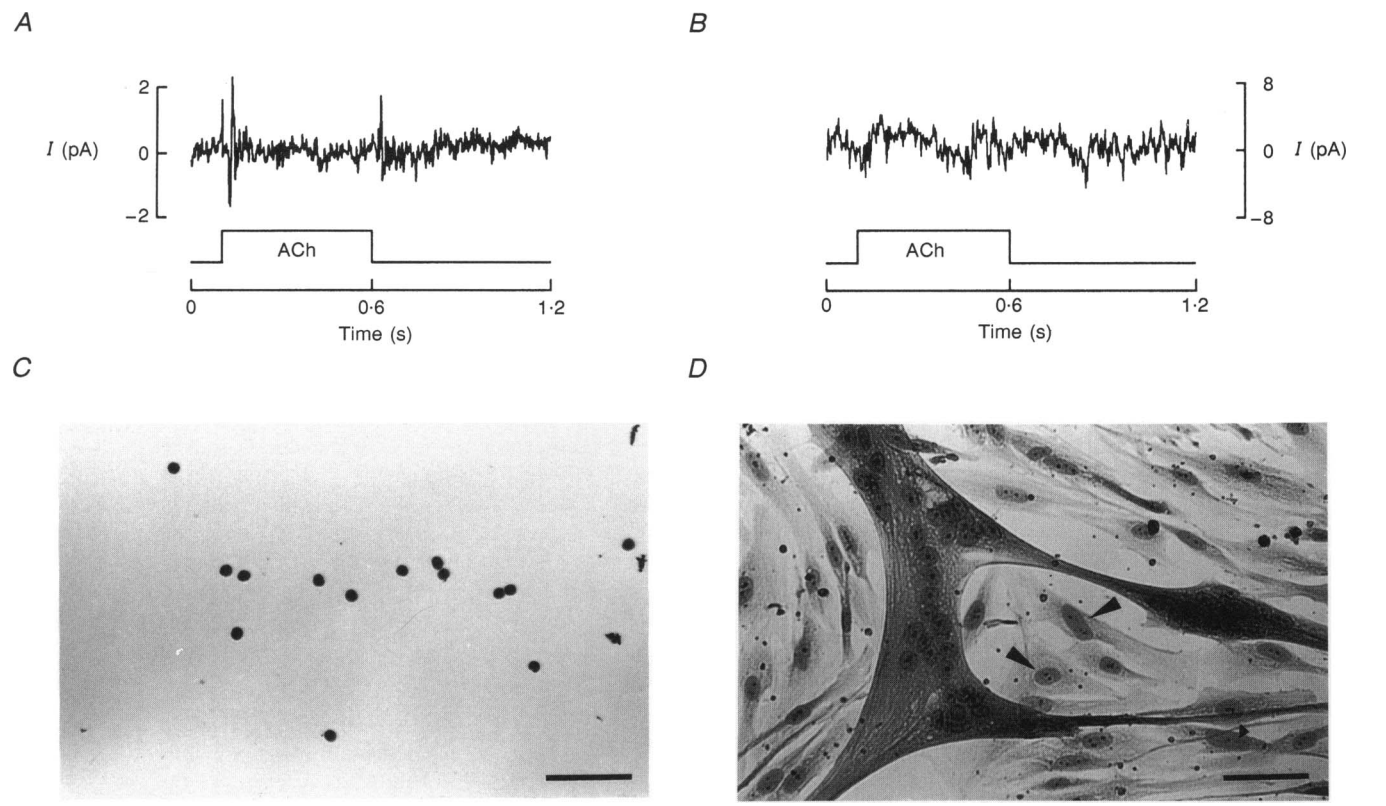

Figure 1. Absence of ACh current in freshly isolated satellite cells and non-fusing myoblasts

A, whole-cell recording of a freshly isolated satellite cell held at −80 mV and exposed for 500 ms to 1 μM ACh. Capacitance of the cell was 6 pF. *B*, whole-cell recording of a non-fusing myoblast kept 3 months in differentiation medium. Same protocol as in *A*. Capacitance was 49 pF. *C*, Haematoxylin-stained freshly isolated satellite cells. Note that these cells are small and that their nucleocytoplasmic ratio is high. *D*, Haematoxylin-stained culture of myotubes and non-fusing myoblasts. Arrowheads point to 2 typical non-fusing myoblasts. Scale bars represent 50 μm.

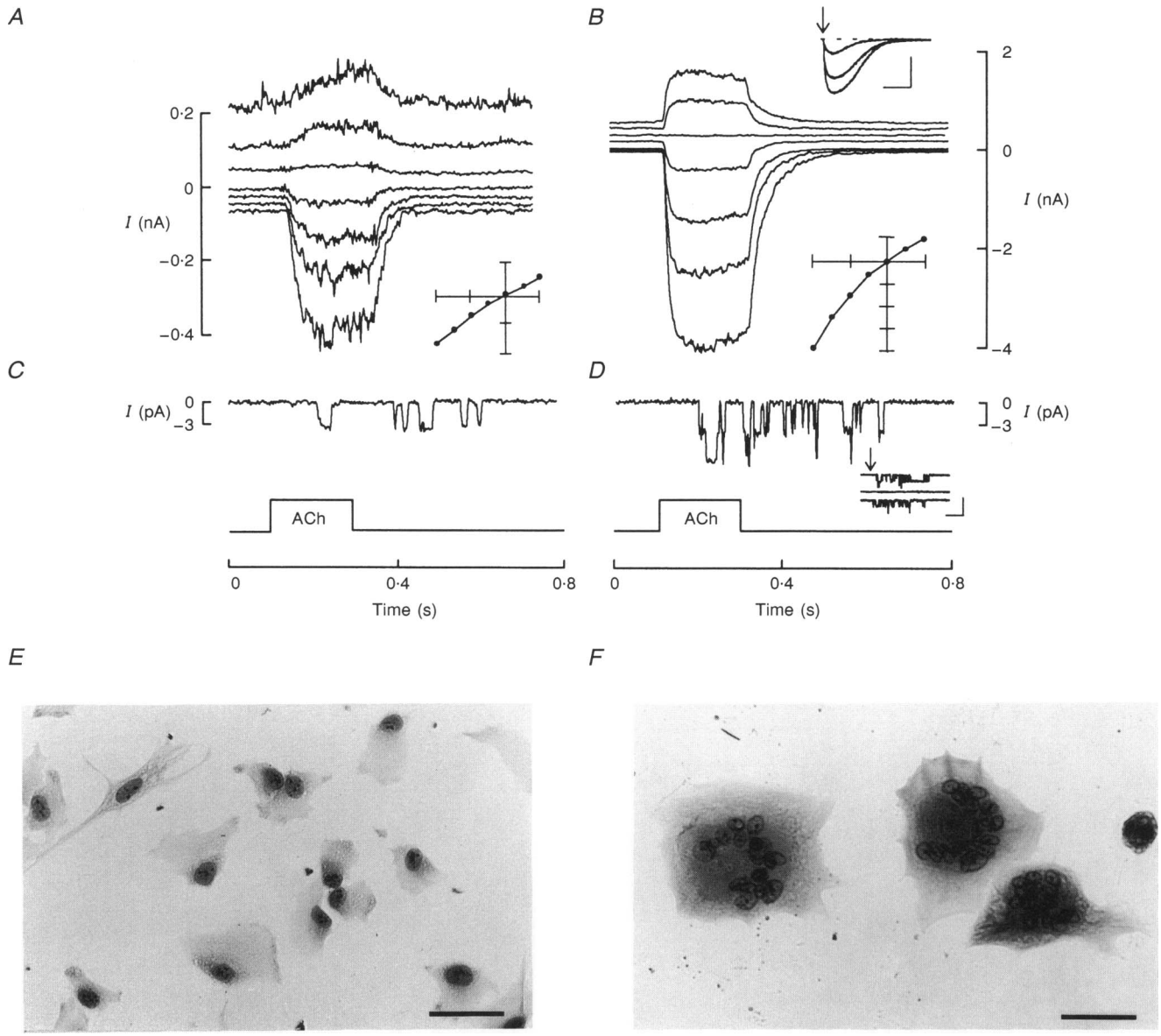

Figure 2. ACh-activated whole-cell and single-channel currents in mononucleated proliferating myoblasts and multinucleated myotubes

A, whole-cell recording from a proliferating myoblast (14 pF) held at voltages between −80 mV and +40 mV (20 mV increments) and exposed for 200 ms to 1 μM ACh. Inset: maximum ACh current plotted as a function of membrane potential (the total length of the abscissa and ordinate was 120 mV and 0·6 nA, respectively). Reversal potential was −3 mV. *B*, whole-cell recording of a myotube (84 pF). Same protocol as in *A*. Lower inset: current–voltage relationship is illustrated (the total length of the abscissa and ordinate was 120 mV and 5 nA, respectively). Reversal potential was 0 mV. Upper inset: ACh-activated whole-cell currents (50 ms pulse of 1 μM ACh) recorded in a myotube held at −80 mV. ACh was pressure-applied at the arrow. The larger, smaller and middle inward currents were recorded in control, 3 μM curare and control (wash) conditions, respectively. Scale bars represent 5 s and 2 nA. *C*, outside-out patch excised from a 36 pF proliferating myoblast. The patch was held at −80 mV and exposed to 1 μM ACh for 200 ms. *D*, outside-out patch excised from a 270 pF myotube; same protocol as in *C*. The inset illustrates single-channel openings elicited by 200 ms pulses of 1 μM ACh (beginning at the arrow) and recorded in control (upper trace), 3 μM curare (middle trace) and control (wash) conditions (lower trace). Scale bars represent 200 ms and 10 pA. The two photographs show examples of Haematoxylin-stained proliferating myoblasts (*E*) and myotubes (*F*). The spherical shape of the myotubes is due to exposure to trypsin before recording (see Methods). Scale bars represent 50 μm.

cells examined. As seen in Fig. 2*A*, ACh induced an inward current at negative potentials and an outward current at positive potential, as expected for a current carried mainly by sodium and potassium ions.

When the proliferation medium was replaced by the differentiation medium, 70–80% of the myoblasts fused and formed multinucleated myotubes (Fig. 2*F*). Figure 2*B* shows ACh currents in a myotube held at various membrane potentials. We found that all myotubes ($n = 11$) expressed large ACh currents (Table 1). Note that we deliberately chose to record from small myotubes to reduce space-clamp artifact. This may explain why the ACh current densities we observed are not as large as in fully mature myotubes.

Curare, an antagonist of the nAChR, reduced ACh currents (Fig. 2*B*, upper inset). When 3 μM curare was added to the superfusion solution, the ACh current was reduced by 75%. The effect was reversible (the middle current trace in the inset was obtained during recovery).

Figure 2*C* and *D* illustrate ACh-activated single-channel currents recorded at −80 mV in a proliferating myoblast (*C*) and in a myotube (*D*). The density of nAChR is low in proliferating myoblasts and ACh-activated channels were only observed in two out of twenty patches. The inset of Fig. 2*D* shows that ACh-induced single-channel openings can be suppressed by curare. A total of five curare applications (2–5 μM) were done during either whole-cell ($n = 2$) or outside-out recordings ($n = 3$). All curare applications drastically reduced ACh currents.

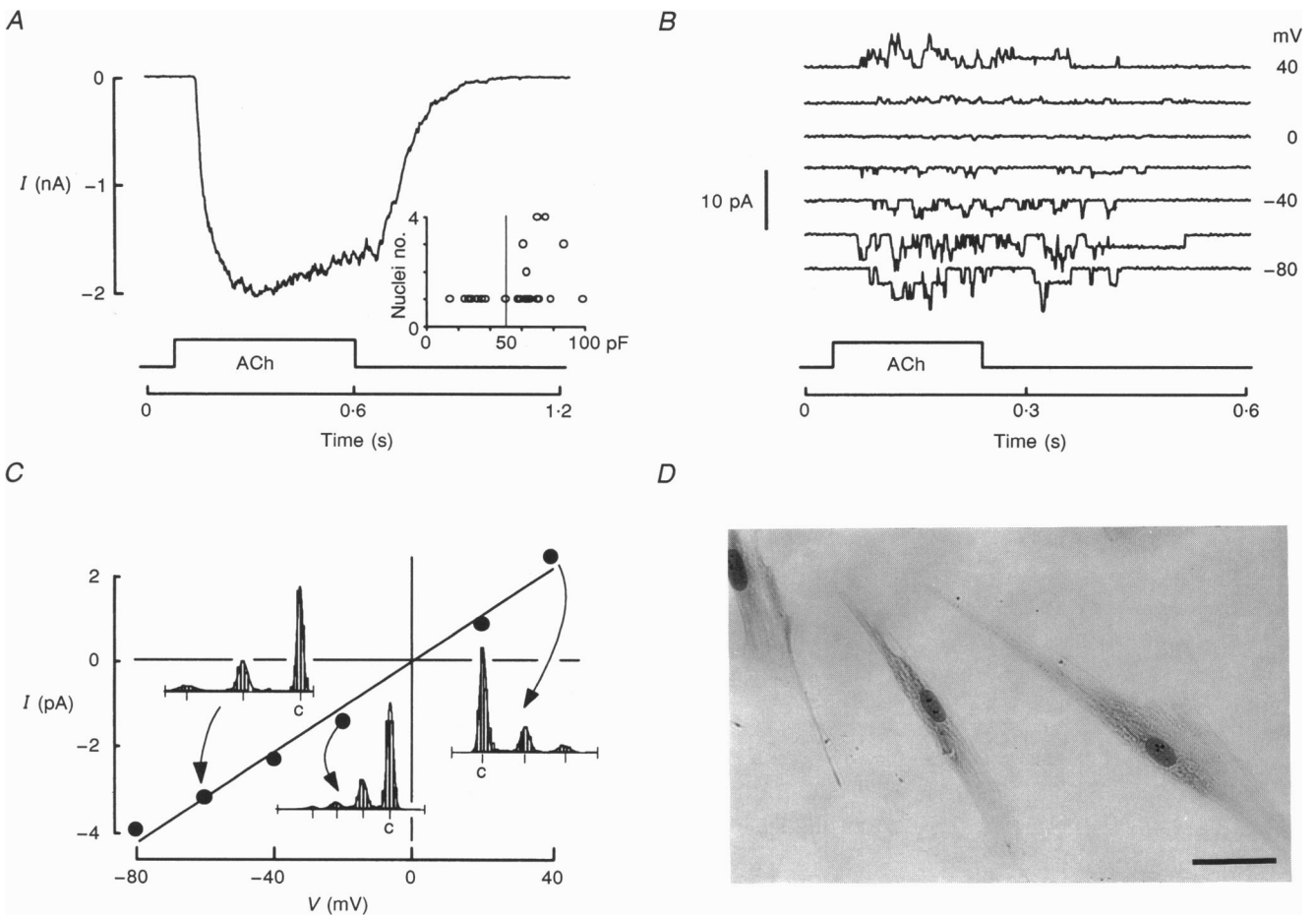

Figure 3. ACh-activated whole-cell and single-channel currents in a prefused myoblast

A, whole-cell recording of a prefused myoblast held at −80 mV and exposed for 500 ms to 1 μM ACh. Capacitance of the cell was 12 pF. Inset: relationship between cell capacitance and number of nuclei in prefused myoblasts (see Methods). *B*, outside-out patch excised from the same prefused myoblast as in *A*. The patch was held at various voltages between −80 and +40 mV (20 mV increments) and exposed for 200 ms to 1 μM ACh. *C*, single-channel current amplitude plotted as a function of the membrane potential. Data points were fitted using a linear regression and the reversal potential was +1 mV. Three examples of current amplitude histograms are shown as insets (c, closed state). The total base length of each histogram represents 8 pA. *D*, Haematoxylin-stained prefused myoblasts. Scale bar represents 50 μm.

Prefused myoblasts

We observed that ACh currents were absent in freshly isolated satellite cells and small in proliferating myoblasts. On the other hand, ACh currents were much larger in myotubes. We wondered whether this upregulation of the expression of ACh-sensitive channels preceded or followed myoblast fusion. As it is not easy to visually assess the precise status (mononucleated, mononucleated with electrical coupling, or multinucleated just fused (Rash & Fambrough, 1973)) of individual cells in the very dense population of fusing cells, we used the following strategy. Proliferating myoblasts were seeded at a very low density (see Methods) and exposed to differentiation medium for 4 days. The idea was that this procedure should generate fusion-competent mononucleated myoblasts that would not be able to fuse because of the large distance between the cells. We called these cells prefused myoblasts.

A first observation in prefused myoblasts was that ACh currents were present in almost all cells tested (93%, $n = 15$). The expression of nAChR in prefused myoblasts is close to that of myotubes and much higher than that of proliferating myoblasts (Table 1). A second observation was that the ACh current density recorded in prefused myoblasts was significantly much larger than that of proliferating myoblasts and not significantly different from the current density of myotubes (Table 1). Thus, even though prefused myoblasts were still mononucleated cells, the ACh current expression of these cells was comparable to that of multinucleated myotubes.

Figure 3*D* shows Haematoxylin-stained prefused myoblasts kept in the differentiating medium for 4 days. Whole-cell (Fig. 3*A*) and single channel recordings (Fig. 3*B*) in a prefused myoblast are illustrated. The current–voltage relationship of single-channel ACh current is shown in Fig. 3*C*.

The ACh receptor agonist carbachol increases the rate of myoblast fusion

Table 1 summarizes the mean ACh current density and other characteristics of cells at the five developmental stages studied. Note that nAChR expression does not seem to be related to myoblast size at any stage of differentiation. Freshly isolated satellite cells are much smaller than non-fusing myoblasts, and neither cell type expressed ACh current. In addition, the average cell capacitance of proliferating myoblasts is almost identical to that of prefused myoblasts despite the large difference in ACh current density in these two cell types.

As already mentioned, a marked and significant increase of ACh current density occurs in prefused mononucleate myoblasts, suggesting that nAChR may play a role in the process of fusion. Thus, we examined whether fusion of human myoblasts could indeed be affected by a non-hydrolysable cholinergic agonist, carbachol.

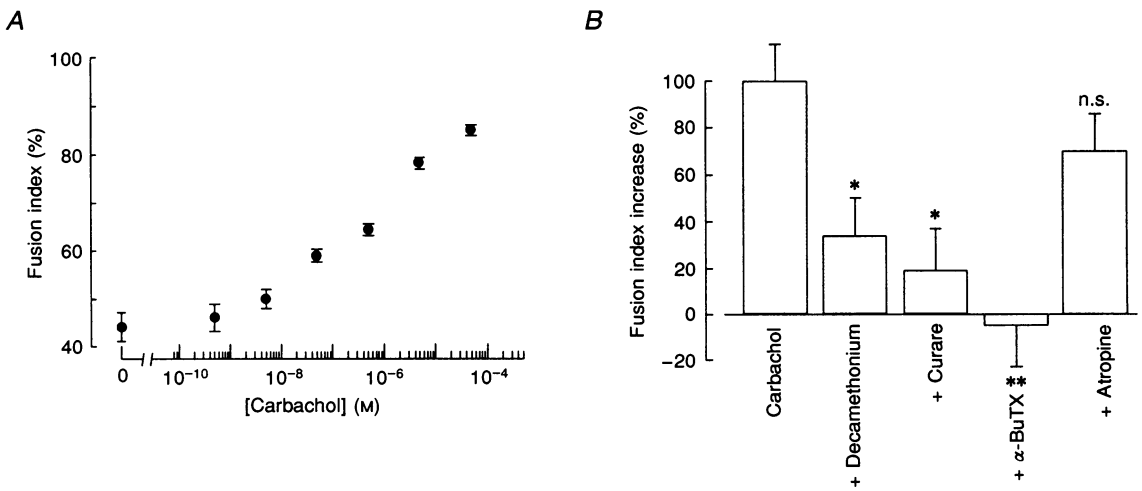

Figure 4. Carbachol increases the rate of myoblast fusion by activating nAChR

A, relationship between fusion index and carbachol concentration. Myoblasts were cultured in differentiation medium supplemented with increasing concentrations of carbachol. The fusion index was assessed at day 5. *B*, effects of nicotinic antagonists (5 μM α-bungarotoxin (α-BuTX), 5 μM curare, 5 μM decamethonium) and of a muscarinic antagonist (2 μM atropine) on the increase in the fusion index induced by 2 μM carbachol. (No obvious toxic effect of the compounds was observed when tested on the proliferation rate of myoblasts.) Two separate experiments were done with each antagonist and the results were pooled. Fusion indexes were assessed after 3–4 days in differentiation medium (in each experiment, the fusion index in the presence of carbachol was normalized to 100%). Error bars represent the fluctuation of the fusion indexes between microscope fields. Results of Student's t test calculations (with respect to carbachol): * $P < 0{\cdot}01$, ** $P < 0{\cdot}001$ and not significantly different (n.s.).

Proliferating myoblasts were cultured in differentiation medium containing increasing concentrations of carbachol, and the fusion index was evaluated after 5 days (see Methods). As shown in Fig. 4*A*, the addition of carbachol to the differentiation medium led to a dose-dependent increase in the fusion index. In this experiment, the fusion index at 5 days was 44% in the control condition and 85% in the presence of 50 μM carbachol, which corresponds to a 93% increase in fusion. A similar experiment done with another clonal culture also showed a dose-dependent increase in the fusion index (from 53 to 71%). In addition, in another series of four experiments, an increase of $37 \pm 14\%$ of the fusion index was observed after 5 days when the differentiation medium was supplemented with 5 μM carbachol. It is worth pointing out that if carbachol speeds up the fusion process, the steady-state fusion index is not affected (data not shown).

As carbachol is able to activate both nicotinic and muscarinic receptors, specific antagonists were added to the differentiation medium in order to determine the type of receptor involved. Figure 4*B* illustrates the result of experiments in which four antagonists were tested: α-bungarotoxin, curare, decamethonium and atropine. To facilitate comparisons between experiments, in each experiment the carbachol-induced increase in fusion index was normalized to 100%. While the muscarinic antagonist atropine (2 μM) failed to significantly affect the carbachol-induced increase, all nicotinic blockers significantly reduced the effect of carbachol, and α-bungarotoxin (5 μM) completely occluded it. Thus, these results indicate that nicotinic rather than muscarinic AChR contribute to the increase in the rate of myoblast fusion.

Fusion indexes in the presence of 2–5 μM carbachol were assessed after 3–5 days in differentiation medium. In the presence of these agonist concentrations, nAChR are known to desensitise with time. Desensitization was small during brief ACh applications (see Table 1) but suppressed a large fraction of the ACh-induced current after a few minutes, and this is illustrated in Fig. 5*A*. A cell was exposed to 5 μM carbachol for 5 min and 2 s periods were recorded every 15 s. It can be seen that after an initial transient, the ACh-induced current progressively decayed and reached approximately 10% of its initial peak value within 5 min. The result in Fig. 5*A*, however, raised another question: in the experiments evaluating the fusion-promoting effect of carbachol, the exposure to the drug lasted several days; was there any ACh current left in the cells tested ?

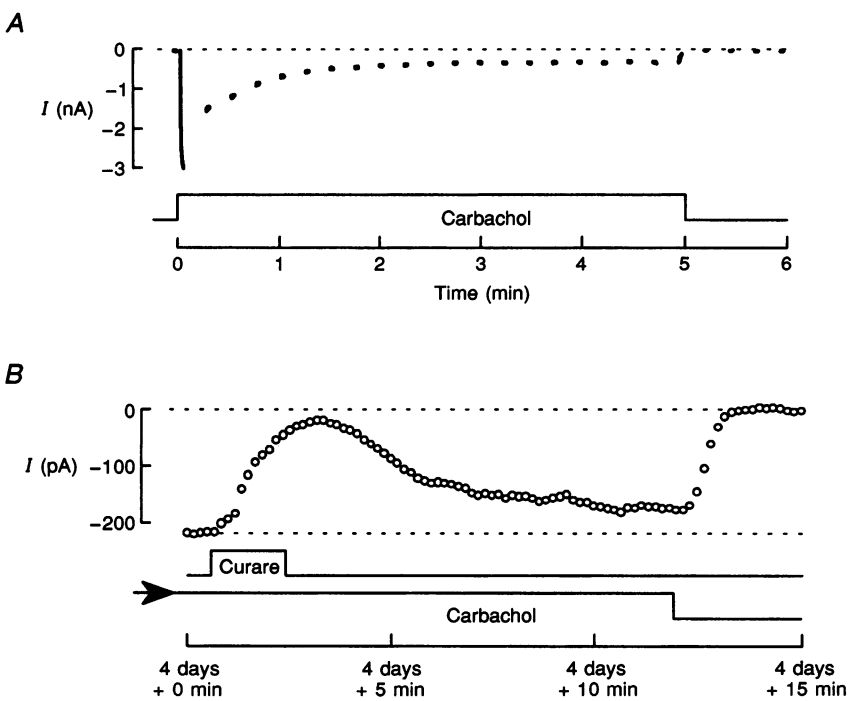

Figure 5. Desensitization of carbachol-activated whole-cell currents

A, whole-cell recording of a myotube (370 pF) held at −80 mV and exposed for 5 min to 5 μM carbachol. A carbachol-activated inward current is represented. Recordings (2 s) were done every 15 s. *B*, whole-cell recording of a myotube (170 pF) held at −80 mV. Carbachol (5 μM) was applied for 4 days before the beginning of the recording and during most of the recording. Curare (5 μM), co-applied with carbachol for 110 s, blocked the carbachol-activated inward current. Leak current (current remaining after removal of carbachol) was subtracted.

Figure 5*B* demonstrates that there remained an ACh current even after 4 days of exposure to carbachol. Myotubes cultured for 4 days in the presence of 5 μM carbachol were put on the recording set-up and superfused with a medium containing carbachol (5 μM). They were then recorded and their potential was held steadily at −80 mV for several minutes to ensure that the holding current, representing the leak current plus the putative ACh-induced current, was stable. Curare (5 μM) was then applied, together with carbachol. A progressive reduction of an inward current could be observed (Fig. 5*B*). The effect of curare was reversible. Finally, when carbachol was removed from the superfusion medium, the inward current decreased to the level of the leak current. This experiment was repeated in three myotubes (203 ± 55 pF) with similar results. On average, the inward current blocked by 5 μM curare was 0·64 ± 0·28 pA pF^{-1}.

This experiment indicates that, despite a substantial desensitization after 4 days in culture, at least part of the effect of carbachol on fusion can be mediated by the flow of ions through opened ACh channels. A further indication that this is the case comes from the observation that decamethonium, an open ACh channel blocker, reduced the effect of carbachol on fusion (see Fig. 4*B*).

DISCUSSION

This study describes the expression of nAChR during the maturation of myoblasts derived from human muscle satellite cells, and shows that the activation of these receptors promotes the formation of myotubes.

The clonal human satellite cell preparation used here, and the culture media (Ham *et al.* 1989; St Clair *et al.* 1992), proved to be convenient for the following reasons. First, clonal cultures eliminated possible complications associated with the presence of non-muscle cells; second, simple changes in the culture conditions allowed us to maintain cells in distinct states of maturation; third, it was possible to study the cells at stages of the maturation process which were otherwise not easily accessible or had not been studied so far (freshly isolated satellite cells, prefused myoblasts, non-fusing myoblasts).

Freshly isolated satellite cells, which we assume have properties that resemble those of satellite cells *in vivo*, did not respond to ACh. When they were cultured in conditions promoting proliferation, these cells divided actively but expressed little or no ACh sensitivity. However, when the cells were transferred to a culture medium that induced fusion, ACh sensitivity was markedly enhanced. Interestingly, the myoblasts that did not fuse in these fusion-promoting conditions were insensitive to ACh.

In a separate set of experiments, we found that activation of nAChR by a cholinergic agonist, carbachol, increased the rate of myoblast fusion, an effect that could be blocked by nAChR antagonists.

Taken together, these results suggest that the expression of nAChR is linked to the maturation of myoblasts derived from satellite cells and that their activation accelerates the process of myoblast fusion and thereby promotes muscle formation.

ACh sensitivity of myoblasts derived from postnatal satellite cells in human and other preparations

The presence of nAChR in postnatal mononucleated myogenic cells has been reported in several preparations. Recordings from satellite cells attached to intact skeletal muscle fibres indicated that they were insensitive to ACh, but when isolated and placed in culture, they acquired a sensitivity to ACh after 48–72 h (Bader *et al.* 1988). Using fine-tip microelectrodes, Eusebi & Molinaro (1984) reported that replicating myoblasts isolated from 2-month-old mice responded to ACh application by a depolarization. Cossu *et al.* (1987), using both patch-clamp and α-bungarotoxin-binding techniques, concluded that replicating mouse myoblasts expressed nAChR.

Our observation that freshly isolated satellite cells were insensitive to ACh is in good agreement with the study done on rat satellite cells still attached to muscle fibres (Bader *et al.* 1988). However, our findings in proliferating human myoblasts differed from those of previous studies done in mice or rats. In human proliferating myoblasts, we found that only half the cells were sensitive to ACh, whereas, in mice and rats, all proliferating myoblasts were sensitive to ACh (Eusebi & Molinaro, 1984; Cossu *et al.* 1987; Bader *et al.* 1988). One of the major differences between the studies done on human and mouse or rat myoblasts is that mouse or rat myoblasts could not be kept in a culture condition that maintained them in a steady proliferating stage, as such a medium has not yet been developed. Indeed, mouse and rat myoblasts fuse and form myotubes as soon as the culture becomes confluent. As a consequence, a fraction of the proliferating myoblasts recorded in these cultures was probably already more mature (prefused stage) than human replicating myoblasts in our cultures. It is the ability to prevent or trigger the onset of differentiation (fusion) in human myoblast cultures that allowed us to describe the striking difference of nAChR density between proliferating and prefused myoblasts.

In the present study, only one size of ACh-activated conductance which corresponds to the embryonic nAChR conductance (Table 1) was observed at all stages of human myoblast maturation (for reviews see Brehm & Henderson, 1988; Lingle, Maconochie & Steinbach, 1992). Channel open time probability distributions were similar in prefused myoblasts and in myotubes. A slightly longer open time

was observed in proliferating myoblasts, but both sets of values are within the range of open times reported for embryonic nAChR (Table 1).

The rate of myoblast fusion is increased by nAChR activation

Myoblast fusion is an essential mechanism in muscle fibre formation and repair. Studies of the fusion mechanism have been almost exclusively done with embryonic myoblasts or with myogenic cell lines (for a review see Wakelam, 1989). It must be recalled, however, that satellite cells and embryonic myoblasts may not be equivalent cell populations (Eusebi & Molinaro, 1984; Cossu & Molinaro, 1987) and that both of these cell types may be distinct from muscle cell lines.

Since the work done by Shainberg, Yagil & Yaffe (1969), it is known that myoblast fusion requires the presence of calcium ions. More recent studies of fusion in embryonic chick myoblasts (Entwistle, Zalin, Bevan & Warner, 1988*a*; Entwistle *et al.* 1988*b*) suggested that fusion was initiated by a membrane depolarization leading to an increase in intracellular calcium concentration, presumably through voltage-dependent calcium channels. Whether this mechanism also applies to human myoblasts derived from satellite cells remains to be verified. However, experiments done in our laboratory revealed that 3 μM of the calcium channel antagonist nifedipine, which blocks 90% of the calcium current in human myotubes, did not affect the carbachol-induced fusion of myoblasts (data not shown).

Using nicotinic and muscarinic AChR antagonists we concluded like Entwistle *et al.* (1988*b*), that the increase of myoblast fusion observed in the presence of carbachol was due to the activation of nAChR. The mechanism by which nAChR activation increases fusion, however, is not yet clarified. ACh-activated channel can lead to a direct increase in intracellular calcium concentration, as this channel is permeant to calcium ions. Using an ion-permeation model, Decker & Dani (1990) calculated that approximately 2% of the total inward current through a nicotinic ACh-activated channel was carried by calcium ions under physiological conditions. We do not know yet whether the increase in human myoblast fusion induced by carbachol is linked to an increase of intracellular calcium concentration. In addition to the well-known effect on the cationic channel, activation of nAChR can induce metabotropic effects. For example, ACh applied on cultured *Xenopus* skeletal muscle reduced an inward calcium current as well as an outward potassium current, presumably through a metabotropic effect of the nAChR (Moody-Corbett & Virgo, 1993). Furthermore, ACh applied on cultured mouse myotubes in the absence of extracellular calcium raised intracellular calcium concentration, and this calcium mobilization was concomitant with inositol 1,4,5-trisphosphate accumulation (Giovanelli, Grassi, Mattei, Mileo & Eusebi, 1991; Grassi, Giovanelli, Fucile & Eusebi, 1993).

In an attempt to distinguish between metabotropic and ionotropic effects of nAChR activation on myoblast fusion, decamethonium was tested. Decamethonium blocks the ACh-activated ionic channel (depolarization or calcium flux through the channel) but not a putative metabotropic effect of the nAChR (Bertrand, Ballivet & Rungger, 1990). In our experiments, decamethonium reduced significantly, but only partially, the carbachol effect compared with the drastic effect of α-bungarotoxin, which blocks both ionotropic and metabotropic effects (Giovanelli *et al.* 1991). This result suggests that the ionic flux through the channel does influence myoblast fusion, but it does not exclude the possibility that a metabotropic process may also be involved.

Cells that do not fuse are insensitive to ACh

Satellite cells may be considered as myogenic stem cells. Stem cells can be defined by their pluripotentiality, i.e. their ability to give rise to a differentiated progeny and their capacity of self-renewal (Lajtha, 1979; Hall & Watt, 1989). Indeed, when a muscle regenerates, a new population of satellite cells is formed and will be found lying on the newly formed muscle fibres (Schultz, 1978; Yao & Kurachi, 1993). This process can be repeated over several cycles of degeneration/regeneration (Schultz & Jaryszak, 1985; Gulati, 1986; Morlet, Grounds & McGeachie,1989).

In our cultures, even after more than 3 months in a differentiation medium that promotes fusion, we could still observe, between the multinucleated myotubes, *mononucleated* cells, which we refer to as non-fusing myoblasts. These cells did not have ACh current. This is not disturbing as there ought to be satellite cells (stem cells) present in these cultures and satellite cells do not have functional nAChR.

As with satellite cells, non-fusing myoblasts isolated from the myotubes were able to resume proliferation and give rise again to both a fusing and a non-fusing progeny (A. Baroffio, M. Hamann, L. Bernheim, M. L. Bochaton-Piallat, G. Gabbiani & C. R. Bader, unpublished observations). Non-fusing myoblasts should therefore have properties similar to freshly isolated satellite cells, i.e. they should stay undifferentiated and they should not respond to ACh. Two other observations are consistent with the view that non-fusing myoblasts may be equivalent to myogenic stem cells. First, non-fusing myoblasts were not labelled by an antibody against sarcomeric α-actin, but were instead expressing desmin (Baroffio *et al.* 1995), like native human muscle satellite cells (Baroffio *et al.* 1993). Second, we found that non-fusing myoblasts, like satellite cells, did not express the voltage-gated potassium current that myoblasts express just before they fuse (Widmer *et al.* 1995).

Can there be a sustained action of ACh?

The efficacy of ACh is linked to a variety of factors that include its concentration and the degree of activation and

desensitization of nAChR. The ACh concentration depends on ACh secretion, on local geometry, and on the presence of active acetylcholinesterase (AChE). It is worth recalling that AChEs have multiple molecular forms and that their synthesis is highly regulated (Grubic, Komel, Walker & Miranda, 1995; for a review see Toutant & Massoulié, 1988). Thus, a modulation of extracellular AChE activity in regenerating muscle might also affect ACh concentration.

Regarding nAChR desensitization, we found that even after several days of exposure to carbachol, myotubes still had a residual ACh-induced current. Could this current carry sufficient amounts of calcium inside the cell to promote fusion? A definite answer cannot be given as the intracellular calcium buffer is not known, but an estimate of the calcium load can. The recorded myotubes had residual currents of 0·64 pA pF^{-1} in the presence of 5 μM carbachol. From this result we can calculate that the residual ACh current in a prefused myoblast of 26 pF (corresponding to a volume of $12{\cdot}5 \times 10^{-12}$ l) would be 17 pA pF^{-1}. We calculated the calcium load induced over 1 h by a steady current of 0·17 pA pF^{-1} (arbitrarily 100 times less, to account for the possibility that the actual ACh concentration *in vivo* may be less than micromolar) assuming that 2% of this current was carried by calcium (Decker & Dani, 1990). We found that there would be an increase of calcium concentration of 5 μmol l^{-1} every hour. Thus, provided there is a source of extracellular ACh in regenerating muscle, the residual current induced by ACh may be able to modify the intracellular calcium concentration of myoblasts sufficiently to stimulate the fusion process.

Could ACh influence fusion *in vivo*?

Our data indicate that *in vitro*, ACh increases the rate of fusion of human myoblasts derived from satellite cells. This result could be of little physiological importance if no ACh was present in a regenerating muscle. However, there may be several sources of ACh in a regenerating muscle. First, there are indications that denervated skeletal muscle may synthesise and release ACh (Mitchell & Silver, 1963; Miledi *et al.* 1982; Tucek, 1982); second, the Schwann cells of a sectioned nerve can release ACh (Dennis & Miledi, 1974); and third, motoneurones may secrete ACh during neurite elongation (Young & Poo, 1983).

In addition to these possible sources of ACh, we show in a companion paper (Hamann *et al.* 1995) that human myoblasts as well as myotubes synthesise and release a compound that can be hydrolysed by the specific enzyme AChE. We also show in this second paper that cultured myoblasts release a compound which, when applied on a voltage-clamped myotube, is able to activate a current with kinetics and pharmacological characteristics similar to the ACh current described in the present paper. Therefore, it is likely that myogenic cells themselves may be a source of ACh.

These results shed new light on the physiological role of embryonic nAChR. As embryonic nAChR are evenly distributed on the membranes of myotubes and denervated muscle, it was suggested that such a diffuse expression of the receptors might make the muscle receptive to innervation at any point of contact and therefore improve the likelihood of synaptic formation (Brehm & Henderson, 1988). We suggest that another possible physiological role for embryonic nAChR in postnatal human myogenic cells is to accelerate muscle regeneration by increasing the rate of myoblast and myotube fusion.

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