A Role for Acetylcholine Receptors in the Fusion of Chick Myoblasts

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Abstract. The role of acetylcholine receptors in the control of chick myoblast fusion in culture has been explored. Spontaneous fusion of myoblasts was inhibited by the nicotinic acetylcholine receptor antagonists α-bungarotoxin, Naja naja toxin and monoclonal antibody mcAb 5.5. The muscarinic antagonists QNB and n-methyl scopolamine were without effect. Atropine had no effect below 1 μM, where it blocks muscarinic receptors; at higher concentrations, when it blocks nicotinic receptors also, atropine inhibited myoblast fusion. The inhibitions imposed by acetylcholine receptor antagonists lasted for ~12 h; fusion stimulated by other endogenous substances then took over.

The inhibition was limited to myoblast fusion. The increases in cell number, DNA content, the level of creatine phosphokinase activity (both total and muscle-specific isozyme) and the appearance of heavy chain myosin, which accompany muscle differentiation, followed a normal time course. Pre-fusion myoblasts, fusing myoblasts, and young myotubes specifically bound labeled α-bungarotoxin, indicating the presence of acetylcholine receptors. The nicotinic acetylcholine receptor agonist, carbachol, induced uptake of [^{14}C]Guanidinium through the acetylcholine receptor. Myoblasts, aligned myoblasts and young myotubes expressed the synthetic enzyme Choline acetyltransferase and stained positively with antibodies against acetylcholine. The appearance of ChAT activity in myogenic cultures was prevented by treatment with BUDR; non-myogenic cells in the cultures expressed ChAT at a level which was too low to account for the activity in myogenic cultures. We conclude that activation of the nicotinic acetylcholine receptor is part of the mechanism controlling spontaneous myoblast fusion and that myoblasts synthesize an endogenous, fusion-inducing agent that activates the nicotinic ACh receptor.

In the previous paper we reported that agents that probably bring about depolarization of fusion competent myoblasts can initiate myoblast fusion (Entwistle et al., 1988). In all cases, these agents were only effective when calcium ions were able to cross the membrane, suggesting that depolarization may open voltage-gated calcium channels. The resultant entry of calcium ions triggers myoblast fusion.

There is already evidence for prostanoids as an important endogenous stimulus for myoblast fusion (Entwistle et al., 1986), possibly generating depolarization by increasing the permeability to chloride ions (Entwistle et al., 1988). In the course of these experiments we showed that an agonist at the acetylcholine receptor, carbachol, also was able to initiate myoblast fusion, through a separate pathway from that triggered by prostanoids. Acetylcholine receptors appear at high density in the myoblast membrane after myotube formation (see Fambrough, 1979 for review) but are present on chick myoblasts at low density and can be detected either electrophysiologically or by the binding of [^{125}I]α-bungarotoxin (Smilowitz and Fischbach, 1978). In this paper we show that nicotinic acetylcholine receptors play a role in the process of myogenesis in vitro and that myoblasts express choline acetyltransferase and specifically bind antibodies to acetylcholine. A brief report of some of these results has already appeared (Bevan et al., 1985).

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Materials and Methods

Myoblast Cultures

Culture preparation and the schedule for the addition of prostanoid synthesis inhibitors were as described in the previous paper (Entwistle et al., 1987). The schedule for other manipulations of fusion are given in the legends to the appropriate figures. Except where indicated, all reagents came from Sigma Chemical Co. (London, U.K.).

Autoradiography

Autoradiographs of cultures labeled with ^{125}I-labeled α-bungarotoxin were made and processed as described elsewhere (Bevan and Steinbach, 1977; Adams and Bevan, 1985). In brief, α-bungarotoxin was iodinated (^{125}I, Amersham, U.K.) by the chloramine T method and fractionated on a Sephadex G-25 column. The resultant [^{125}I]α-bungarotoxin had an initial specific activity of 170–230 Ci/mmol^{-1}. Cultures were incubated with 10^{-8} M labeled α-bungarotoxin for 60 min at room temperature, followed by extensive washing for 30 min to remove unbound radioligand. In some cases 10^{-4} M carbachol or D-tubocurare was added before and during exposure to labeled α-bungarotoxin to inhibit toxin binding to the acetylcholine receptor. La-

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beled cultures were fixed in 4% glutaraldehyde in phosphate buffer at room temperature for 1 h, washed, dehydrated through a graduated series of methanols, and then air dried. The cultures were coated with a thin film of nuclear gel emulsion (Ilford K2, diluted 1:1 with water at 40°C) and exposed at 4°C for 4-28 d before development.

Assay of Choline Acetyl Transferase Activity

Choline acetyl transferase activity was determined using the method of Fonnum (1975), modified to take account of the low levels of activity pertaining in cultured myoblasts. The dishes were rapidly rinsed twice with ice cold, 50 mM phosphate buffer containing 10 mM EDTA, made up to isotonicity with sodium chloride, at pH 7.4, followed by a rinse in the same solution, but lacking EDTA. Cells were gently scraped from the bottom of the dish with a silicone rubber stopper and harvested in 250 μl of EDTA-free buffer in an Eppendorf tube. The tubes were spun for 4 min at 500 *g* and most of the supernatant removed, leaving a total volume of ∼27 μl. 1 μl of EDTA-free buffer containing either 5 U of acetylcholine esterase or 1 mM physostigmine was then added to each tube. Total protein was assayed in parallel by the micro-assay of Bradford (1976) to assess cell loss during these procedures.

The reaction was started by adding 0.09 μCi [^{14}C]acetyl CoA (Amersham) in 2 μl of EDTA-free buffer containing 1 mM acetyl CoA (Sigma Chemical Co.), 40 mM choline chloride and 2.5% Triton X-100 (vol/vol) which gives a final concentration of choline of 2.7 mM. The Triton ensured efficient cell lysis. The mixture was incubated for 15 min at 37°C and the reaction stopped with 2.5 ml of ice cold EDTA containing buffer at pH 7.0. A 100-μl aliquot of the suspension was taken for protein assay. The remainder received 1 ml of 1% (wt/vol) tetraphenylboron in acetonitryl (BDH) followed by 4 ml of scintillation fluid (Ready-solv, Beckman). It was then vortex-mixed, spun at 3,500 *g* for 10 min, and the aqueous phase removed with a silanized Pasteur pipette. The organic phase was counted in a Beckman Model LS 7500 or LS 1801 liquid scintillation counter. A blank set of dishes was carried through the same procedures to assess the amount of Acetyl CoA partitioned into the organic phase. ChAT activity was calculated from the difference in radioactivity between dishes treated with physostigmine and those treated with acetylcholine esterase. The results are expressed as pmol/min per mgm protein on the basis of the protein content determined in the aliquots taken before counting.

To determine carnitine acetyltransferase activity, 10 mM carnitine (final concentration) was substituted for choline in the assay described above and the reaction stopped with ice-cold EDTA in buffer at pH 3.0 to enhance the entry of acetylcarnitine into the organic phase. The activity in myoblasts was calculated by comparison with a standard curve constructed using pigeon breast carnitine acetyltransferase (Sigma Chemical Co.). If the reaction was stopped with EDTA buffered at pH 7.0, as for choline, the level of activity in the organic phase fell to background levels, confirming that the extraction method normally excludes acetylcarnitine (Fonnum, 1975; Tucek et al., 1978). By contrast, in controls using labeled acetylcholine more than 80% of the initial radioactivity was partitioned into the organic phase; this was abolished by the inclusion of AChE in the incubation medium.

Immunocytochemistry

The appearance of the muscle-specific form of Creatine phosphokinase and of the myosin-heavy chain was detected with antibodies using a double staining technique. A rabbit polyclonal antibody to the muscle specific CPK isozyme was kindly donated by J. C. Perriard (University of Zurich, Zurich, Switzerland); a mouse monoclonal myosin antibody came from G. Dhoot (University of Birmingham, Birmingham, U.K.). The cultures were fixed in 1:1 acetone/methanol at −20°C for 1.5 min, air dried, and stored at 4°C. For assay each dish was preincubated at 37°C with 1 ml PBS, pH 7.4, containing 10% horse serum, rinsed thoroughly with PBS and then incubated for 1 h at 37°C in a high humidity atmosphere with 1:100 dilution of the anti-muscle specific CPK antibody. They were then rinsed thoroughly with PBS and incubated with a fluoresceinated anti-rabbit second layer antibody for 1 h under the same conditions. After further rinsing, the anti-myosin monoclonal antibody (1:100) was applied for 1 h. The myosin specific antibody was rinsed away and a rhodaminated, anti-mouse second layer applied, again for 1 h. After further rinsing the cultures were mounted in anti-fade mountant (Citifluor, City of London Polytechnic, London, U.K.) and viewed at 300× magnification through the appropriate filters in a Zeiss IIIRS microscope equipped for epi-fluorescence. The cytoplasm surrounding 200-300 nuclei from each culture, taken across the radius of the dish, were scored for the number showing positive staining, and the results then expressed as the percentage of nuclei in each dish surrounded by positively staining cytoplasm. Controls using the second layer antibodies alone showed no specific staining. Cultures were scored blind in order to avoid subjective bias.

Immediately pre-fusion and aligned myoblasts (50 h of culture) were stained for acetylcholine with a rabbit polyclonal antibody against glutaraldehyde conjugated acetylcholine (Biosoft, France; Geffard et al., 1985). The cultures were fixed for 1 min in 2% formalin with 0.5 M allyl alcohol and 0.15 M cacodylate buffer, post-fixed in the absence of allyl alcohol for 15 min, and then washed thoroughly with 0.1 M phosphate buffer. They were stained with the ACh antibody (1:100) in phosphate buffer with 0.1% Triton X-100 and 2% FCS (1:200) for 1.5 h, washed, then exposed to biotinylated goat anti-rabbit antibodies (1:200) for 1 h, washed thoroughly, and exposed to FITC-coupled Streptavidin for 1 h, washed thoroughly with phosphate buffer and mounted in anti-fade mountant. No specific staining was observed in controls using second layer antibodies only, when allyl alcohol was omitted from the fixative or if the cells were treated with Triton X-100 before fixation. DNA content and total Creatine phosphokinase activity were determined as described in Entwistle et al. (1986).

Guanidinium Uptake and Efflux

10^{-8} M α-bungarotoxin was added to one set of dishes containing myoblasts 30 h after plating 1 h before uptake measurement began. Immediately before the uptake measurements the culture medium was replaced by 700 μl isotonic-buffered sucrose (290 mM sucrose, 20 mM glucose, 5.4 mM KCl, 10 mM Hepes, set to pH 7.4 with Tris base). At time zero 48 μCi of [^{14}C]guanidinium (specific activity 48 mCi/mmol, Amersham International, U.K.) and sufficient carbachol to bring the final concentration to 10^{-5} M were added to all dishes. 30 s later 4 dishes were taken from each set, rapidly, but gently, rinsed 7 times to remove the loading solution, and 1 ml buffered sucrose containing Triton X-100 (0.1%) added to lyse the cells. Further sets of dishes were similarly treated at 1, 2, and 4 min after the addition of labeled medium. The solution from each dish was taken up into 3 ml of Ready-solv and counted in a liquid scintillation counter. The amounts of radioactivity contained by the cells at each time point were used to construct an uptake curve and the carbachol-induced uptake of guanidinium calculated as the difference between the uptake curves in the absence and presence of α-bungarotoxin.

Guanidinium efflux measurements were made on immediately pre-fusion myoblasts and young myotubes. The cultures were exposed to [^{3}H]guanidinium for 4-12 h to load them to equilibrium. They were rapidly rinsed five times to remove labeled guanidinium from the extracellular space and at time zero 1 ml of unlabeled medium of the same composition as the load solution added to each dish. The wash solution was withdrawn and replaced with 1 ml new solution at 15 s, 0.5- or 1-min intervals and after a steady efflux rate had been established solution containing 10-500 μM D-tubocurare was added to the dish. Four samples were taken in the presence of dTc and the efflux solution then returned to normal medium. At the end of the efflux measurements the cells were lysed by the addition of 1 ml 0.1% Triton X-100 and the lysate counted to provide the radioactivity remaining within the cells. The rate constant (k) was calculated from the ratio of the efflux of radioactivity/min and the appropriate cumulative activity of the cells.

Results

Blocking the Nicotinic Acetylcholine Receptor Delays Myoblast Fusion

When myoblast fusion has been delayed by preventing synthesis of prostanoids, fusion can be induced by the acetylcholine agonist, carbachol. The fusion-inducing effect of carbachol is blocked by the nicotinic acetylcholine receptor blocker, α-bungarotoxin, which does not affect fusion induced by the addition of Prostaglandin E_1 (PGE_1)[1] (Entwistle et al., 1987). Fig. 1 *a* shows that α-bungarotoxin can also prevent spontaneous myoblast fusion, assessed at 54 h of culture, when 40-50% fusion has taken place in controls, in a concentration-dependent manner. Inhibition was obvious at 10^{-9} M and maximal at 10^{-8} M. Cell number and myoblast alignment were unaffected. In keeping with the results

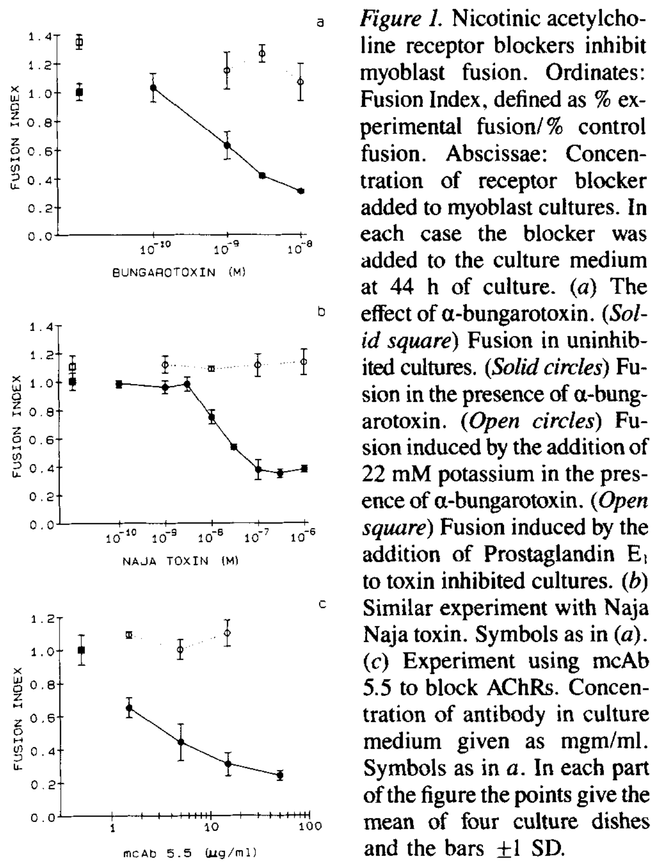


Figure 1. Nicotinic acetylcholine receptor blockers inhibit myoblast fusion. Ordinates: Fusion Index, defined as % experimental fusion/% control fusion. Abscissae: Concentration of receptor blocker added to myoblast cultures. In each case the blocker was added to the culture medium at 44 h of culture. (*a*) The effect of α-bungarotoxin. (*Solid square*) Fusion in uninhibited cultures. (*Solid circles*) Fusion in the presence of α-bungarotoxin. (*Open circles*) Fusion induced by the addition of 22 mM potassium in the presence of α-bungarotoxin. (*Open square*) Fusion induced by the addition of Prostaglandin E_1 to toxin inhibited cultures. (*b*) Similar experiment with Naja Naja toxin. Symbols as in (*a*). (*c*) Experiment using mcAb 5.5 to block AChRs. Concentration of antibody in culture medium given as mgm/ml. Symbols as in *a*. In each part of the figure the points give the mean of four culture dishes and the bars ±1 SD.

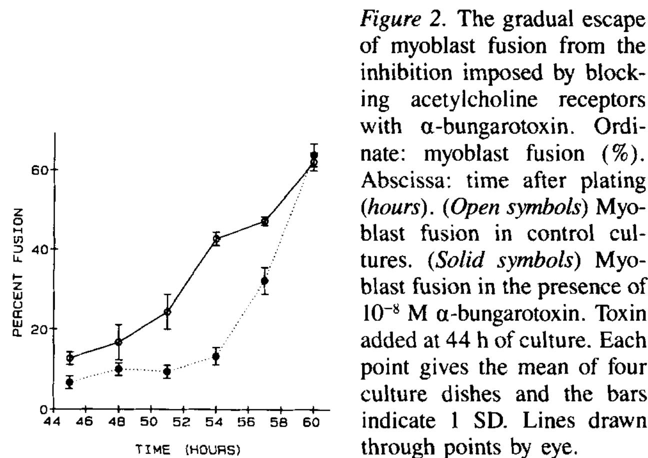


Figure 2. The gradual escape of myoblast fusion from the inhibition imposed by blocking acetylcholine receptors with α-bungarotoxin. Ordinate: myoblast fusion (%). Abscissa: time after plating (*hours*). (*Open symbols*) Myoblast fusion in control cultures. (*Solid symbols*) Myoblast fusion in the presence of 10^{-8} M α-bungarotoxin. Toxin added at 44 h of culture. Each point gives the mean of four culture dishes and the bars indicate 1 SD. Lines drawn through points by eye.

reported in the previous paper, either the addition of PGE_1 (Fig. 1 *a*, *open square*) or raising potassium to 20 mM (Fig. 1 *a*, *open circles*) overcame the block imposed by α-bungarotoxin, initiating fusion at the level observed in uninhibited, parallel cultures (Fig. 1 *a*, *solid square*). In this particular experiment fusion induced by potassium was substantially (fusion index 1.4) greater than spontaneous fusion. This was very unusual. In all other experiments, potassium often caused a small (10–15%) stimulation of fusion, although there was no evidence for the induction of precocious fusion (see Entwistle et al., 1988). The reason for the substantial stimulatory effect of potassium in this experiment is not known. Another snake neuro-toxin, Naja naja toxin, that also binds to nicotinic acetylcholine receptors, was similarly effective in preventing spontaneous fusion of myoblasts (Fig. 1 *b*). Both these snake toxins can bind tightly to sites other than acetylcholine receptors in some cell types (e.g., PC12 cells, Patrick and Stallcup, 1977). Fig. 1 *c*, (*solid circles*) shows that a monoclonal antibody (mcAb 5.5), which binds to and inactivates nicotinic acetylcholine receptors in chick muscle (Goldberg et al., 1983), was also able to inhibit spontaneous myoblast fusion, the block again being overcome by the addition of 20 mM potassium (*open circles*). As controls for an effect of nonspecific antibody binding we tested two other monoclonal antibodies, LBl which also binds to chick myoblasts (Wakelam, M. J. D., personal communication), although not to the nicotinic acetylcholine receptor, and A2E5. At protein concentrations equivalent to those which gave clear inhibitions with antibody mc Ab5.5 there was no inhibition of myoblast fusion by either antibody.

The inhibition of spontaneous fusion achieved by blockers of nicotinic acetylcholine receptors was not complete and a low level of fusion, ~10–15%, took place nevertheless (see Fig. 1 *a*). This low level of spontaneous fusion is similar to that found when myoblast fusion is delayed by inhibiting prostanoid synthesis (Entwistle et al., 1986). Myoblasts eventually escaped from the inhibition of fusion imposed by blocking the nicotinic acetylcholine receptor. Fig. 2 compares the time course of myoblast fusion in normal cultures (*open circles*) with the time course of fusion in the presence of 10^{-8} M α-bungarotoxin (*solid circles*). At 54 h of culture fusion in toxin-containing cultures was still less than 15%, compared with 42% in the parallel, untreated cultures. However by 57 h, fusion in toxin treated cultures had reached 32% and by 60 h it was no longer any different from the untreated control. This suggests that during the inhibition there is a build-up of fusion-inducing agents other than those activating the nicotinic acetylcholine receptor that eventually overcomes the toxin-induced block. A similar escape occurred during treatment with Naja naja toxin and was noted previously during inhibition of prostanoid synthesis (Entwistle et al., 1986). Repeated dosing with any of the acetylcholine receptor blockers was unable to prolong the time course of the block, suggesting that the escape did not arise because of toxin coming off the cells. Furthermore, treatment with both prostanoid synthesis inhibitors and blockers of the acetylcholine receptor was unable to prevent the eventual initiation of spontaneous myoblast fusion. As noted previously (Entwistle et al., 1988), myoblast fusion must then proceed by yet another route.

Table I. Muscarinic Acetylcholine Antagonists Have No Effect on Myoblast Fusion

Antagonist	Concentration	Fusion index
Atropine	10^{-8} M	0.99 ± 0.04
N-Methyl scopolamine	10^{-9} M	0.97 ± 0.07
	10^{-8} M	0.83 ± 0.07
QNB	10^{-8} M	1.09 ± 0.06
	10^{-6} M	1.03 ± 0.05

All values give the mean ±1 SD of four dishes. In each case the drug was added after 24 h in culture and the assay carried out at 52 h of culture. A second dose of QNB 10^{-6} was added at 42 h.

1. *Abbreviations used in this paper*: PGE_1, Prostaglandin E_1; CPK, creatine phosphokinase.

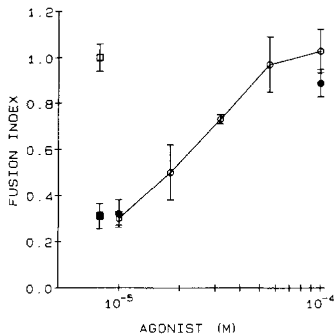


Figure 3. Curare can act as a fusion-inducing agent in chloroquine-inhibited cultures. Ordinate: fusion index. Abscissa: agonist concentration (*M*). Open square: control fusion in parallel, uninhibited cultures. (*Solid square*) Fusion in chloroquine-inhibited cultures. (*Open circles*) Fusion in the presence of carbachol. (*Solid circles*) Fusion in the presence of D-tubocurare. Carbachol or D-tubocurare added at 54 h of culture. Each point gives the mean ±1 SD of four culture dishes except for control where 8 dishes were used. Line drawn through points by eye.

Table I shows that antagonists to muscarinic acetylcholine receptors (atropine, quinuclidinylbenzilate [QNB], and N-methyl scopolamine) had little or no effect on myoblast fusion when applied at concentrations which would be expected to block specifically muscarinic receptors. However, atropine concentrations above 1 μM, where block of nicotinic acetylcholine receptors is known to occur (Feltz et al., 1977), did suppress myoblast fusion. In 5 experiments (20 dishes) control fusion was 45.2 ± 2.8% (±1 SEM). In the presence of 1 μM Atropine, fusion fell to 14.5 ± 4.3% (±1 SEM, $n = 5$, 20 dishes). Taken together these results confirm that nicotinic, rather than muscarinic, acetylcholine receptors are involved.

If activation of nicotinic acetylcholine receptors is involved in myoblast fusion, then D-tubocurare, a well known antagonist of acetylcholine at the neuromuscular junction, might be expected to prevent the spontaneous fusion of myoblasts. However no inhibition of fusion was found with 10^{-3} or 10^{-6} M D-tubocurare. Fig. 3 shows that, in chloroquine-inhibited cultures, D-tubocurare (*solid circles*) proved to be an effective fusion-inducing agent, equivalent to carbachol (*open circles*). The agonist action of D-tubocurare could be prevented by α-bungarotoxin (10^{-8} M). This apparently anomalous finding might be explained if the drug acts as an agonist to the acetylcholine receptor on chick myoblasts, as described in rat myoblasts and young myotubes (Trautman, 1982; Takeda and Trautman, 1984). Experiments to test this possibility directly were made by following the efflux of guanidinium ions, which can permeate the ACh receptor activated channel, in the absence and presence of 10–500 μM D-tubocurare. On some occasions a significant efflux of guanidinium ions was induced by dTC and reduced by α-bungarotoxin, consistent with an agonist action at the ACh receptor. The variability of the response could arise if the agonist action was short-lived and followed by antagonistic effects. However these experiments do not exclude the possibility that dTC induces myoblast fusion by some completely separate mechanism.

Nicotinic Acetylcholine Receptor Blockers do not Inhibit Myoblast Differentiation

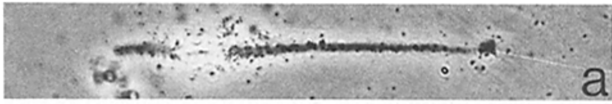
Although nicotinic antagonists delayed myoblast fusion, the block could be completely overcome by raising extracellular potassium or the addition of PGE_1. This suggests that the inhibition operates at the level of myoblast fusion, rather than reflecting a general effect on myoblast differentiation. This is supported by measurements of several other properties of myoblasts that are known to change in the course of normal myogenesis in vitro. Table II shows that at 54 h of culture cholinergic antagonists had no effect on the changes in the cell number, DNA content, level of total creatine phosphokinase (CPK) activity, appearance of the muscle specific CPK isozyme and the appearance of myosin heavy chains

Table II. Nicotinic Acetylcholine Receptor Antagonists Block Myoblast Fusion but Have No Effect on Cytodifferentiation

	Control		Bungarotoxin 54 h	Naja toxin 54 h	Atropine 54 h
	24 h	54 h			
			10^{-8} M	*10^{-7} M*	*10^{-5} M*
Percent fusion	none detected	42.8 ± 2.5	113.7 ± 2.7	14.8 ± 1.5	14.6 ± 1.5
Cell No. × 10^{-3}/sq cm	16.7 ± 1.5	40.8 ± 6.1	43.6 ± 6.1	38.5 ± 4.0	45.1 ± 6.1
DNA μg/sq cm	67 ± 15	104 ± 4.0	119 ± 9.0	123 ± 17	116 ± 6
Total CPK nmol/ min/μg DNA	10.3 ± 3.1	44.8 ± 10.8	38.6 ± 5.3	44.6 ± 6.4	40.8 ± 5.5
+ve M-CPK (%)	12.6 ± 8.0	61.4 ± 5.6	65.7 ± 1.1	ND	ND
+ve myosin (%)	13.5 ± 4.5	64.5 ± 0.3	64.6 ± 1.7	ND	ND

ND = not determined. Values given/sq cm refer to sq cm of culture dish. All values given mean ±1 SD of four parallel dishes. They represent examples drawn from at least 3 measurements of each parameter, carried out in various combinations.
The apparent discrepancy between the increase in cell number and the increase in DNA content between 24 and 54 h of culture arises because at 24 h many cells have replicated their DNA, but have not yet undergone cytokinesis.

Figure 4. Pre-fusion myoblasts contain α-bungarotoxin–binding sites indicating the presence of acetylcholine receptors. (*a* and *b*) Phase-contrast (*a*) and bright-field (*b*) images of single bipolar myoblast after 30 h in culture. Note silver grains over the myoblast indicating specific binding of labeled α-bungarotoxin. (*c* and *d*) Another single myoblast at 30 h in culture. (*e* and *f*) Phase-contrast (*e*) and bright-field (*f*) images of field showing both myoblasts and fibroblasts. Note only myoblasts show silver grains reflecting α-bungarotoxin binding. (*g* and *h*) Field of aligning myoblasts at 48 h in culture to show specific toxin binding. Note some myoblasts (*arrows*) have very low binding levels. (*i* and *j*) Field of myotubes at 144 h in culture showing dense labeling with α-bungarotoxin and at least one hot spot.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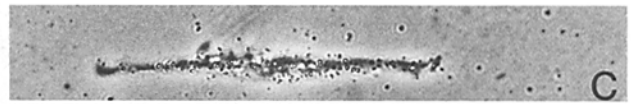
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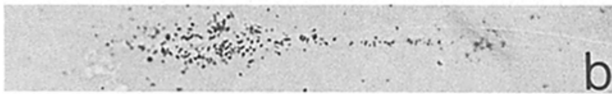
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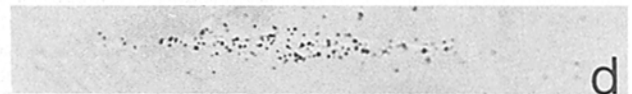
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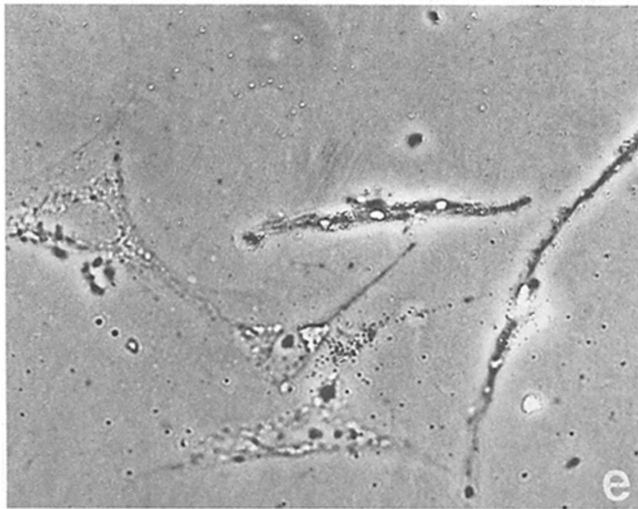
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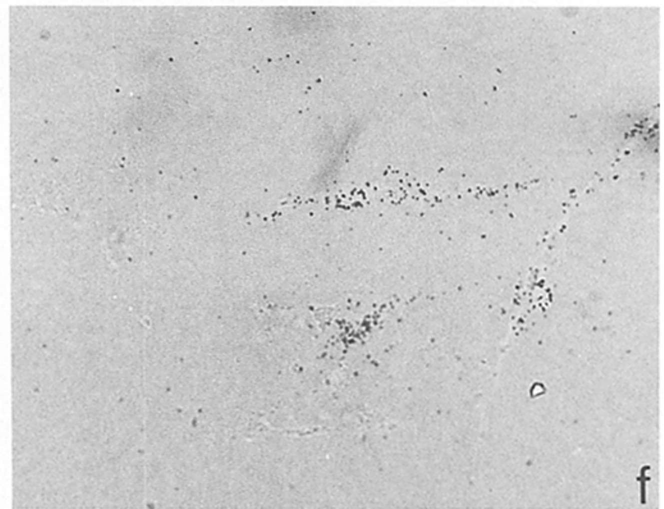
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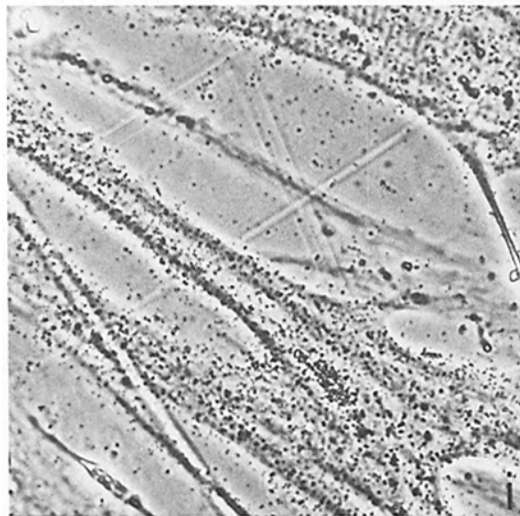
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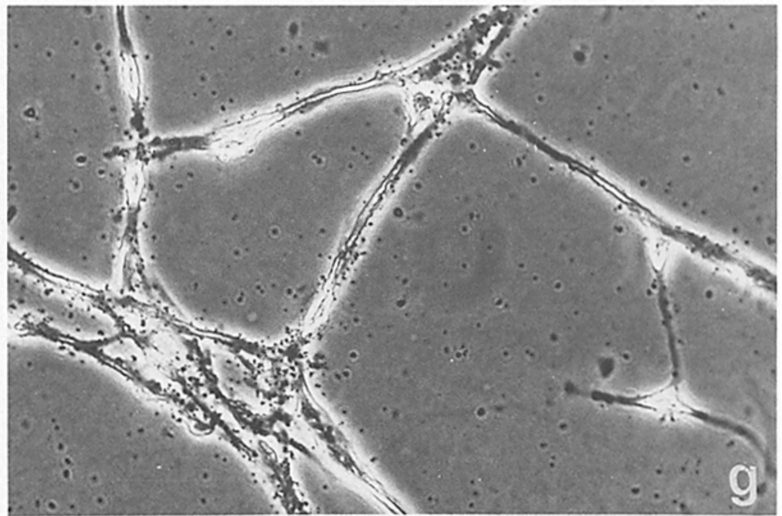
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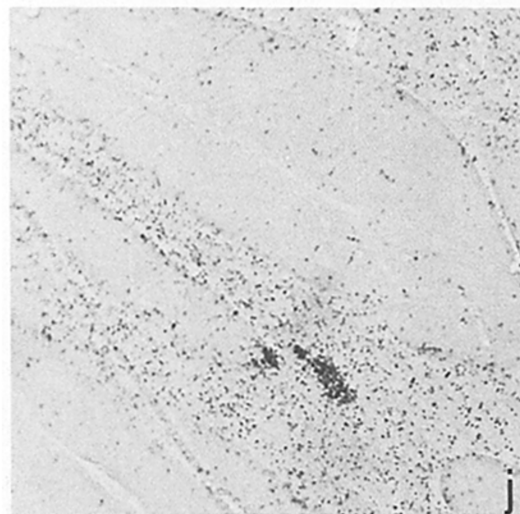
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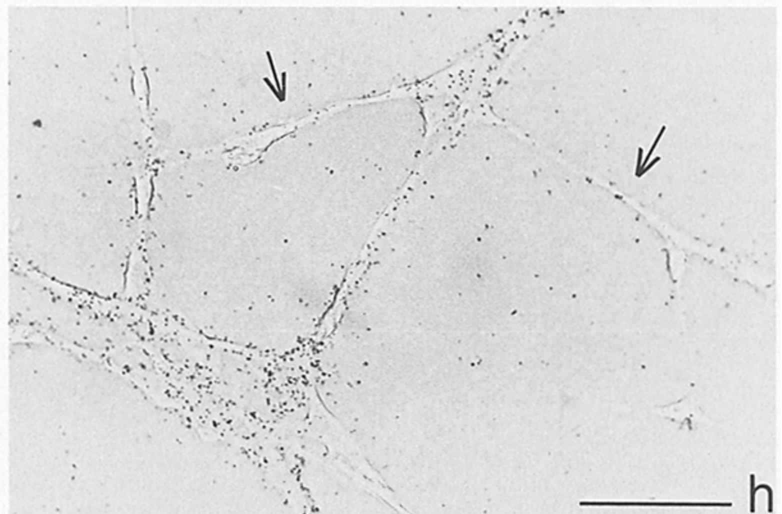
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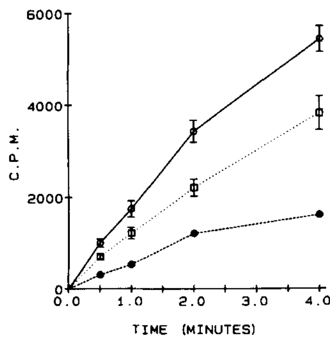


Figure 5. Carbachol induces uptake of [^{14}C]guanidinium in fusing myoblasts. Ordinate: dpm × 10^{-3} per dish. Abscissa: time after beginning of uptake (*minutes*). (*Open circles*) Uptake of guanidinium in the presence of 10 μM carbachol. (*Open squares*) Uptake of guanidinium in presence of 10 μM carbachol and 10^{-8} M α-bungarotoxin to block uptake through acetylcholine receptors. (*Solid circles*) Carbachol induced uptake of guanidinium, calculated as the difference in uptake in the presence and absence of α-bungarotoxin.

that accompany normal myoblast differentiation. When assessing the proportion of nuclei surrounded by cytoplasm positively stained for the CPK isozyme or myosin heavy chains, no specific precautions were taken to differentiate between fused and unfused myoblasts since cultures containing predominantly unfused cells nevertheless showed a high proportion of positive staining.

Myoblasts Possess α-Bungarotoxin–binding Sites and Functional Acetylcholine Receptors

The appearance and distribution of α-bungarotoxin–binding sites, was determined by autoradiography of myoblast cultures exposed to ^{125}I-labeled α-bungarotoxin. Fig. 4 shows sample autoradiographs made from myoblast cultures before fusion begins (30 h), at the beginning of the main burst of spontaneous fusion (48 h), and after the formation of myotubes (144 h). Bipolar, single cells in both 30- and 48-h cultures bound α-bungarotoxin (Fig. 4, *a–f*), although fibroblasts did not. At 30 h 23.7 ± 2.8% (mean ±SEM; 3 dishes) of solitary bipolar cells showed clear α-bungarotoxin–binding, while a higher percentage (53.3 ± 4.0%; mean ±SEM; 3 dishes) of aligned myoblasts were labeled. At 48 h aligning myoblasts (Fig. 4, *g* and *h*) again showed specific binding of labeled toxin, although not all cells were labeled. After 144 h in culture, when extensive chains of myotubes had formed, both diffuse toxin binding and hot spots were evident (Fig. 4, *i* and *j*). Comparison of the level of binding sites in myotubes (Fig. 4 *j*) with that in single myoblasts (Fig. 4, *b* and *d*) shows that some myoblasts possess a high density of toxin-binding sites, similar to that in formed myotubes. The binding of α-bungarotoxin could be prevented if either carbachol (10^{-3} M) or D-tubocurare (10^{-3} M) was included in the medium during labeling.

For acetylcholine to be a plausible fusion-inducing stimulus during myogenesis in culture, it is necessary to show that the myoblast membrane contains functional nicotinic acetylcholine receptors. We therefore tested whether carbachol was able to produce an increase in the sodium permeability of pre-fusion myoblasts. The uptake of [^{14}C]guanidinium, which has been shown to permeate through acetylcholine receptors channels in skeletal muscle (Dwyer et al., 1980), was used to indicate the uptake of sodium. The experiments were made on myoblast cultures at 30 h in culture. Fig. 5 shows the uptake by myoblasts of [^{14}C]guanidinium evoked by 10^{-5} M carbachol in the absence (*open circles*) and presence (*open squares*) of 10^{-8} M α-bungarotoxin. The cells were preincubated with the toxin for 1 h before measurements began. The solid circles give the carbachol induced uptake through the α-bungarotoxin sensitive pathway. These observations suggest that the pre-fusion myoblast membrane contains functional acetylcholine receptors.

Myoblasts Express Choline Acetyltransferase Activity and Bind ACh Antibodies

The finding that blocking nicotinic acetylcholine receptors can delay the spontaneous fusion of cultured myoblasts raises the possibility that some cells in the cultures, possibly myoblasts themselves, synthesize and release acetylcholine or a similar agonist. To test whether myoblasts might be able to synthesize acetylcholine, cultures of myoblasts and myotubes were assayed for the presence of the synthetic enzyme choline acetyltransferase (ChAT) using a modification of an existing procedure (see Materials and Methods).

Control experiments showed that the endogenous level of carnitine acetyltransferase activity, which might complicate estimates of ChAT activity (Rossier, 1977; Goodman et al., 1984), in myoblasts after 50 h of culture was 378 ± 75 μU/culture dish (416 ± 8 μU/mgm protein, $n = 6$). The synthesis of acetylcarnitine was not sensitive to acetylcholine-

Table III. The Levels of Choline Acetyl Transferase Activity in Myogenic Cultures

Time in culture	Activity			ChAT activity pmol/ min per mgm protein
	Blank	+Physo	+ACHE	
		dpm (n)		
Experiment 1				
50 h*	281 ± 32 (6)	607 ± 75 (5)	292 ± 61 (6)	3.3 ± 0.84
96 h*	277 ± 17 (6)	1187 ± 73 (6)	678 ± 96 (6)	2.68 ± 0.7
144 h*	299 ± 64 (6)	1242 ± 106 (6)	494 ± 85 (6)	5.58 ± 1.4
Experiment 2				
50 h*	326 ± 22 (6)	536 ± 48 (5)	287 ± 43 (5)	3.2 ± 0.7
BUDR treated 50 h	447 ± 59 (6)	781 ± 77 (6)	651 ± 72 (6)	1.04 ± 1.1
Fibroblasts	280 ± 19 (6)	480 ± 84 (6)	383 ± 33 (6)	0.9 ± 0.9

Each figure gives the mean ±1 SD with the number of replicates in parentheses.
* Activity in the presence of physostigmine significantly greater than in the presence of AChE ($P < 0.05$; Mann-Whitney Test. Cells were plated at a density of 8 × 10^4/sq cm BUDR (1 × 10^{-5} M) added at 6 h of culture and again at the medium change (24 h).

esterase, as seen previously (Blum et al., 1971; Hamprecht and Amano, 1974). When 1 mU of pigeon breast muscle carnitine acetyltransferae (twice the endogenous level at 50 h) was incubated with 2.7 mM choline as in the standard assay (see Materials and Methods), the activity in the organic phase was no different from background. 10 mU carnitine acetyltransferase gave detectable levels of radioactivity (∿5 times background).

The outcome of ChAT assays is shown in Table III. In 50-h cultures of myoblasts, when fusion is just beginning, the organic phase clearly contained detectable radioactivity, which was abolished completely by the inclusion of AChE in the incubation medium. This suggests that at 50 h the cells possess ChAT activity. Cultures treated with bromodeoxyuridine (BUDR) showed a low level of activity, which was not sensitive to incubation with AChE, suggesting that ChAT levels were much reduced. At 96 h, by which time fusion should be maximal, the myoblasts showed an increased level of AChE sensitive activity, suggesting that ChAT activity was maintained although acetylation of choline by increased levels of carnitine acetyltransferase cannot be ruled out (see Goodman et al., 1984). The esterase insensitive activity increased also, which would be consistent with a concomitant rise in carnitine acetyltransferase activity or endogenous levels of carnitine. Older cultures containing myotubes (144 h) retained significant levels of AChE sensitive activity, while there was little increase in the activity expressed in the presence of AChE. Table III shows also that ChAT activity in cultures of fibroblast-like cells, derived from myogenic cultures by repeated sub-passaging, was substantially lower than in the myoblast cultures, making it unlikely that the ChAT activity in the myoblast cultures came from contaminating fibroblasts.

These measurements indicate that myoblasts at the time of fusion and, possibly, myotubes possess choline acetyltransferase activity.

Although it is likely that the radioactive compound extracted into the organic phase in these experiments was acetylcholine, this remains to be proved. As an alternative, we tested whether cultures at 50 h, which contain bipolar myoblasts, aligning myoblasts, and the earliest myotubes, specifically bound antibodies to acetylcholine. Fig. 6 shows photographs of bipolar myoblasts and aligned myoblasts which show specific binding of an antibody to ACh (*a–d*), compared with binding in controls in which allyl alcohol was omitted from the fixative (*g*) or Triton X-100 was applied before fixation (*e* and *f*). Epithelial cells showed no specific binding of ACh antibodies (*arrows* in *a* and *b*). Thus it is likely that bipolar myoblasts and aligning myoblasts not only possess ChAT activity, but also synthesize acetylcholine.

Discussion

This paper provides evidence that myoblasts in culture are provoked to fuse and form myotubes by activation of the nicotinic acetylcholine receptor; an agonist at the ACh receptor could be an endogenous fusion-inducing substance. Thus spontaneous fusion can be delayed by blocking nicotinic acetylcholine receptors, pre-fusion myoblasts possess α-bungarotoxin–binding sites and activation of myoblast acetylcholine receptors produces an increase in membrane permeability to guanidinium ions, which are known to permeate the ACh channel in the same way as sodium. Furthermore pre-fusion myoblasts specifically bind antibodies to ACh and express the enzyme ChAT, which is associated with the synthesis of acetylcholine (Moolenar and Polak, 1980; Miledi et al., 1982). The ionic basis of myoblast fusion induced by activation of the acetylcholine receptor was discussed in the previous paper (Entwistle et al., 1988) and is not reconsidered here.

The identification of the nicotinic, rather than the muscarinic, acetylcholine receptor as the mediator of myoblast fusion rests on comparison of the ability of known nicotinic and muscarinic acetylcholine receptor antagonists to inhibit spontaneous myoblast fusion. Of the muscarinic antagonists, atropine was able to delay myoblast fusion, but only at concentrations well above those at which it blocks muscarinic receptors in other tissues, when it is known also to bind to nicotinic receptors (Feltz et al., 1977). Further support comes from the specific binding of labeled α-bungarotoxin to pre-fusion myoblasts and the finding that toxin binding could be prevented by carbachol and D-tubocurare.

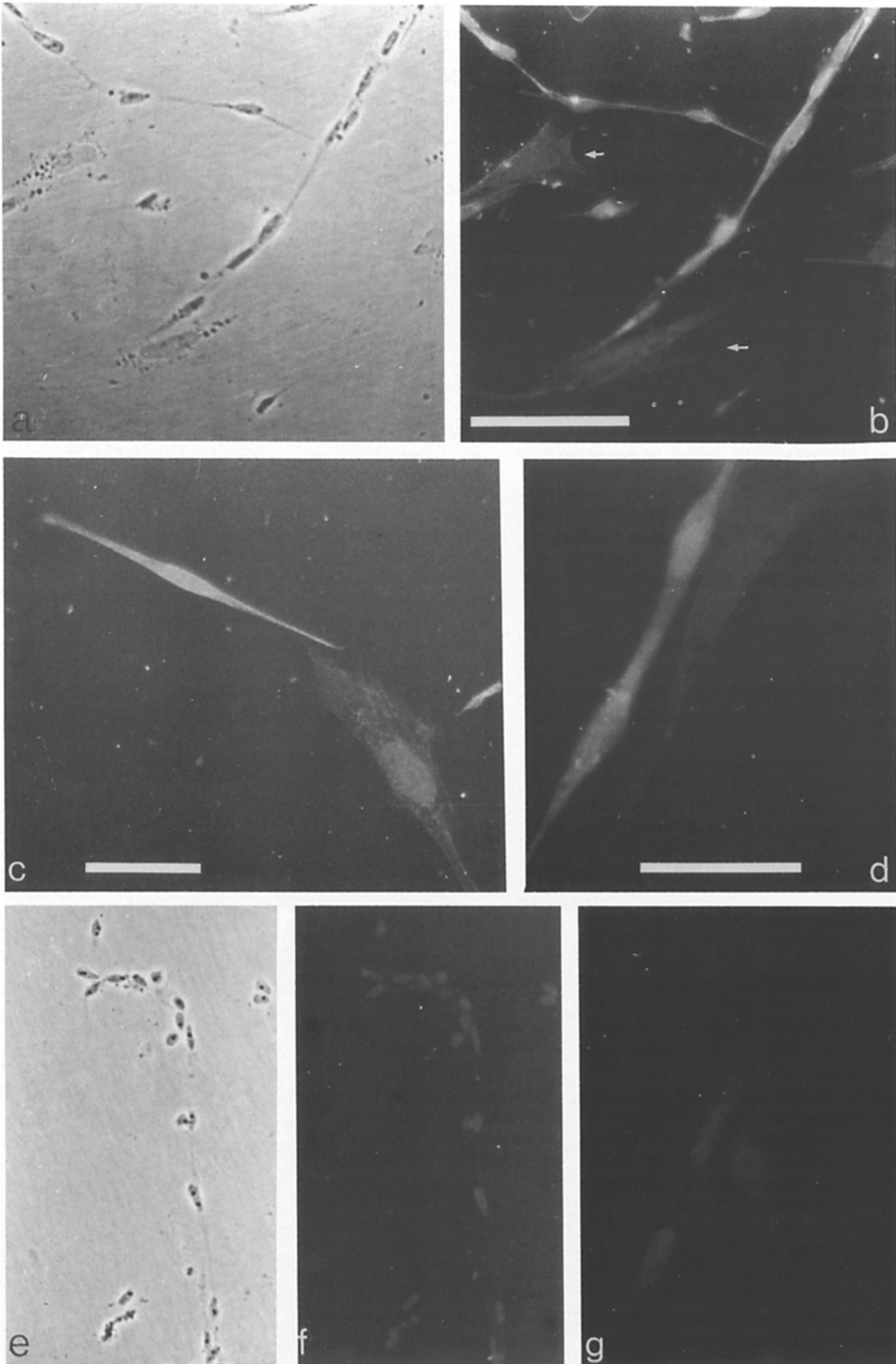
The inhibition of myogenesis imposed when the nicotinic acetylcholine receptor is blocked is restricted to the prevention of myoblast fusion. Cell division and the alignment of myoblasts in preparation for fusion are unaffected, as is the increase in creatine phosphokinase activity and the appearance of myosin, which are both considered to reflect early steps in muscle differentiation. The block is therefore very similar to that achieved by inhibiting prostanoid synthesis (Entwistle et al., 1986). These observations support a growing body of evidence that the link between myotube formation and the differentiation of the phenotypic properties of muscle can be dissociated. Thus blocking the acetylcholine receptor, inhibiting prostanoid synthesis and lowering external calcium to ∿1/10 of its normal level (see Entwistle et al., 1988) all seem to affect the fusion process alone. Manipulation of the ionic environment also can either prevent or promote fusion depending on the particular conditions chosen (see Entwistle et al., 1988). The ability to separate these two processes in a variety of ways should aid analysis of the underlying control mechanisms.

Carbachol induced an α-bungarotoxin sensitive, uptake of guanidinium ions in fusing myoblasts. This is the counterpart to the demonstration that sodium ions must be present in the medium for the acetylcholine agonist to act as a fusion-inducing agent (Entwistle et al., 1988) and reinforces the suggestion that depolarization of the myoblast membrane is an integral part of the initiation of myoblast fusion (Entwistle et al., 1988).

Both myoblasts and myotubes express enzyme activity that is probably, although not certainly, choline acetyltransferase. The partition of ACh into tetraphenylboron at pH 7.0 has been shown to be specific (Fonnum, 1975; Tucek et al., 1978) and myoblasts were labeled specifically with an antibody to acetylcholine. It is unlikely that contaminating fibroblasts are the source of acetylcholine in the cultures because they contain much less ChAT than myoblasts and did not bind the ACh antibody. In addition, fibroblasts form a relatively small proportion of the cells in the cultures, since 80% of cells are destined to form myotubes, setting the maximal likely contamination with non-myogenic cells at 20%.

The Control of Spontaneous Myoblast Fusion

Evidence has been accumulating that prostanoids may be in-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volved in myoblast differentiation and fusion (Zalin, 1977; Entwistle et al. 1986; David and Higginbotham, 1981). Part of this evidence rests on the finding that spontaneous fusion of myoblasts in culture is substantially delayed when prostanoid synthesis is inhibited. The observation that blocking the acetylcholine receptor also substantially delays spontaneous fusion poses a number of questions about the control of myoblast fusion both in vitro and in vivo. The two pathways can be activated independently. However the effective concentration for PGE_1-induced fusion is increased when the acetylcholine receptor is blocked (Entwistle et al., 1988) and preventing activation through either pathway is sufficient to delay spontaneous fusion in vitro (this paper and Entwistle et al., 1988). This suggests that cultured myoblasts produce subthreshold amounts of more than one fusion-inducing agent and the two pathways act synergistically during spontaneous fusion. One of these agents is probably a prostanoid (Zalin, 1977; Entwistle et al. 1986; David and Higginbotham, 1981). The other acts at the nicotinic ACh receptor and might be acetylcholine itself, although the present results do not allow its unequivocal identification. The situation during normal development is more difficult to define. Prostaglandins and prostanoid-binding sites have been detected in differentiating myoblasts (Hausman et al., 1986; Zalin, 1987).

Both innervated and denervated frog and rat muscle have been shown to synthesize acetylcholine (Miledi et al., 1977, 1978, 1981), although its functional role has not been identified. The presence of ChAT activity in young myotubes would not, therefore, be totally unexpected. However, the demonstration of ChAT activity does not, in itself, mean that either myoblasts or myotubes are synthesizing acetylcholine. The activity could be masked functionally and only released from the inactive form during the lysis necessary to carry out the assay. The finding that myoblasts bind specifically an acetylcholine antibody is more convincing. Muscle synthesized acetylcholine may persist for some time and could assist the accretion of myoblasts during myotube formation.

The earliest motoneurons enter the muscle mass very early in development (Landmesser, 1978) and may secrete acetylcholine during neurite elongation (Young and Poo, 1984). Thus the present findings raise the interesting possibility that neurons have a role in myoblast fusion. It will be interesting to know whether young myotubes retain the ability to synthesize ACh after the arrival of the motoneuron, and whether the fusion-inducing effect of any ACh released from muscle is additive with that released by innervating motoneurons. However any involvement of neuronally released acetylcholine cannot be obligatory since differentiated muscles, albeit rather small in size, can be found in limbs deprived of motor innervation before the motor nerves have entered the limb. It is perhaps more plausible to suppose that neuronal activation of acetylcholine receptors may assist the continual accretion of myoblasts into myotubes and in the formation of secondary myotubes. Muscles formed in the absence of innervating motoneurons may be small not only because of the absence of electrically driven motor activity, but also because fewer myotubes are formed in these circumstances.

One consistent feature of the results is the gradual escape of the myoblasts from inhibitions of fusion imposed either by reducing prostanoid synthesis or by including blockers of nicotinic acetylcholine receptors in the culture medium. This suggests that myoblasts are able to fall back on yet another fusion-inducing mechanism which comes into play when fusion is greatly delayed. The present results shed no light on this auxillary mechanism, but it could be related to the presence of membrane receptors to ATP (Kolk and Wakelam, 1983; Hume and Honig, 1986) and we now have evidence to suggest that this is the case (Entwistle and Bevan, manuscript in preparation). The presence of three, apparently independent mechanisms each of which leads to myoblast fusion, implies that this event is of such importance during myogenesis that natural selection has ensured a very large safety factor.

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Figure 6. Myoblasts and young myotubes specifically stain with acetylcholine antibodies. (*a–d*) Phase contrast photograph (*a*) and companion fluorescent image (*b*) of microscope field containing single and aligning myoblasts together with epithelial cells. Note specific staining of bipolar myogenic cells with the ACh antibody, which does not stain cells with an epithelial morphology (*arrows*). (*c* and *d*) High power photographs of a single, stained bipolar myoblast lying next to an unstained epithelial cell (*c*) and part of a chain of aligned, and labeled myoblasts next to an unstained epithelial cell (*d*). (*e*) Phase contrast photograph and (*f*) companion fluorescent image of a microscope field of cells stained with ACh antibody after treatment with Triton X-100 to permeabilize the cells prior to fixation. (*g*) Fluorescence photograph of cells stained with ACh antibody after fixation in the absence of allyl alcohol so that the cross-linking reaction does not take place. Note absence of specific staining in *e–g*. Bars, 25 μm.

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