THE EFFECT OF LOCAL ANAESTHETICS AND BARBITURATES ON MYOGENESIS AND MYOTUBE INTEGRITY IN RAT SKELETAL MUSCLE CULTURES

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SUMMARY
Several cationic anaesthetics and barbiturates including bupivacaine, lignocaine and pentobarbital reversibly inhibit myoblast fusion in rat primary muscle cultures. The same drugs also cause disruption of myotube structure, which is normally complete within 2 h; fusion of myoblasts and reformation of myotubes takes place on removal of the drugs. The disruption of myotubes caused by tertiary amine anaesthetics observed in vitro appears to mimic muscle fibre disruption caused by the same drugs in vivo. The effects of temperature, culture age, medium constitution and calcium flux on anaesthetic-induced myotube breakdown have been examined.

INTRODUCTION
When injected into muscle in vivo some tertiary amine anaesthetics are myotoxic (Benoit & Belt, 1970). In general, clinically used concentrations appear to be destructive to skeletal muscle, but not to other local tissues (Benoit & Belt, 1972). Injection of 0.5% (15 mM) bupivacaine into rat skeletal muscle causes breakdown of the muscle fibres during the first day after injection. (Benoit & Belt, 1970, 1972). The damaged fibres have pyknotic nuclei, lack myofibrils and transverse striations and 24 h after injection the cytoplasm of damaged fibres has fragmented (Benoit & Belt, 1970). Regeneration begins rapidly, cells resembling myoblasts increase in number between the first and second days and within 3 days few single nucleated myoblasts are seen. By 16 days muscle fibres are normal apart from centrally placed nuclei in most of the fibres.

In this type of rapid regeneration, also seen after systemic administration of anti-malarial drugs (Benoit & Belt, 1972), myoblastic elements appear to arise as single cells from within the damaged portions of fibres rather than by budding from healthy fibre ends at the periphery of the lesion. Regeneration of the muscle and of the motor end plate is independent of acetylcholine release and muscular activity (Jirmanova & Thesleff, 1976).

In this study we have investigated the effects of anaesthetic action under the simpler

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conditions of primary culture. Myoblasts from chick embryo or new born skeletal muscle differentiate in culture into postmitotic myoblasts (Holtzer, 1970). These aggregate and fuse to form multinucleate myotubes which are striated and contract spontaneously. The differentiation and fusion is accompanied by accelerated synthesis of proteins specific to mature muscle, and the system has proved valuable in studying many of the events connected with muscle differentiation (Fambrough & Rash, 1971; Holland & McLennan, 1976; Paterson & Prives, 1973; Paterson & Strohman, 1972; Turner, 1975). Fusion can be blocked by Ca\(^{2+}\) deprivation (Shainberg, Yagil & Yaffe, 1969; van der Bosch, Schudt & Pette, 1973; Schudt & Pette, 1976), neuraminidase, phospholipases A and C (Schudt & Pette, 1976; Nameroff & Munar, 1976), lectins (Den, Malinzak, Keating & Rosenberg, 1975), ethidium bromide (Brunk & Yaffe, 1976), cytochalasin B or D (Holtzer, Strahs & Biehl, 1975; Miranda & Godman, 1973) and aspirin or indomethacin (Zalin, 1977).

We have examined the effects of several tertiary amine anaesthetics and barbiturates on both myoblast fusion and on fully formed myotubes in newborn rat skeletal muscle cultures. We show that local anaesthetics of the tertiary amine type and barbiturates can prevent myoblast fusion and cause myotube breakdown. Fusion of myoblasts takes place on removal of the drugs.

**MATERIALS AND METHODS**

**Cell culture**

Cells from the thigh muscle of 1-day-old Wistar-Furth or Sprague-Dawley rats were disaggregated and plated by the method of Yasin, van Beers, Bulien & Thompson (1976) omitting the collagenase. The seeding density was 2 or 3 x 10^6 cells per 3-mm dish (Sterilin) in Dulbecco's Modified Eagle's Medium (DMEM) containing 2 % chick embryo extract (CEE) (Flow labs.) and 10 % foetal calf serum (FCS) (Gibco Biocult). The medium was changed every 2 or 3 days after plating. Cells reached confluence after 3 days and fusion began after 4 days, continuing for a further 3 days. By 10 days striations were observed and contracting myotubes frequently seen.

In some experiments the medium was replaced with minimal Eagle's Medium plus 20 mM HEPES (MEM/HEPES), phosphate-buffered saline (PBS) or Liebovitz L15 medium (Gibco Biocult).

**Effects of local anaesthetics and barbiturates**

Drugs were dissolved in sterile water to a concentration of 50 times the final concentration required. Bupivacaine HCl which was a gift from Duncan Flockart Ltd, lignocaine (Astra, Watford), cocaine (May & Baker, Dagenham), mepivacaine (Pharmaceutical Manuf. Co., Epsom), pentobarbital (Macarthy's Ltd, Romford) and barbitone (BDH, Poole) were used. 40 μl of prepared solution was added to 2 ml of medium in the culture. The effect of local anaesthetics and barbiturates on myotubes was determined by counting the number of myotubes or myotube branches in 10 fields of view under 10 × magnification, and calculating the mean. Cultures were also fixed and stained to determine the percentage of the total nuclei present in myotubes. They were fixed in absolute methanol for 5 min, stained with 20 % May-Grünwald for 10 min and in 10 % Giemsa for 3 min, rinsed in distilled water and covered with a thin layer of glycerol. The total nuclei and the nuclei in myotubes were counted under 40 × magnification. A minimum of 1000 nuclei were counted per culture. Cell death was estimated by trypan blue staining. The cultures were stained with a solution containing trypan blue:0.9 % saline (3:1 v/v).

The effect of temperature on the behaviour of the local anaesthetics was tested by placing
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cultures in a sealed container through which a steady stream of 5% CO\textsubscript{2}/95% air was passed. The temperature was kept constant using a thermostat-controlled convector heater.

To investigate whether calcium flux could affect the action of bupivacaine on myotube structure, cultures were treated with 5, 10 and 100\textmu M of the divalent cation carrier A23187 (Lilly; kindly supplied by Dr B. D. Gomperts) either 30 min before or concurrently with 12 mM bupivacaine.

Photography

Photographs were taken using a Pan F or HP\textsubscript{4} film at an exposure time of 4 s.

RESULTS

Effects of anaesthetics on fusion: General effects

Several nerve-blocking agents, including the tertiary amine anaesthetics bupivacaine, lignocaine, mepivacaine and cocaine and the anionic barbiturates pentobarbital and barbitone inhibited myoblast fusion (Table 1). Cultures were treated with the anaesthetics on day 3. Half of the cultures were fixed and stained on day 6 to determine the degree of fusion; the medium of the remaining cultures was replaced by drug-free medium and the subsequent behaviour of the cultures followed.

Table 1. Inhibition of fusion by tertiary amine anaesthetics and barbiturates

<table>
<thead>
<tr>
<th>Drug concentrations, mM</th>
<th>Myotubes per field of view (x 10)</th>
<th>Nuclei per field of view (x 40)</th>
<th>% nuclei in myotubes</th>
<th>Approximate decrease in % fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19 ± 2</td>
<td>99 ± 4</td>
<td>17 ± 5 ± 5.5</td>
<td>—</td>
</tr>
<tr>
<td>Bupivacaine 0.6</td>
<td>8 ± 1</td>
<td>55 ± 4</td>
<td>2 ± 0.7 ± 0.7</td>
<td>83</td>
</tr>
<tr>
<td>Lignocaine 1</td>
<td>25 ± 2</td>
<td>110 ± 3</td>
<td>5 ± 1.9 ± 1.9</td>
<td>67</td>
</tr>
<tr>
<td>Mepivacaine 1</td>
<td>0 ± 1</td>
<td>69 ± 5</td>
<td>3 ± 1.5 ± 1.5</td>
<td>78</td>
</tr>
<tr>
<td>Cocaine 0.6</td>
<td>150 ± 6</td>
<td>107 ± 2</td>
<td>6 ± 2 ± 2</td>
<td>63</td>
</tr>
<tr>
<td>Pentobarbital 1</td>
<td>7 ± 2</td>
<td>91 ± 6</td>
<td>5 ± 1.7 ± 1.7</td>
<td>70</td>
</tr>
<tr>
<td>Barbitone 0.6</td>
<td>17 ± 2</td>
<td>81 ± 3</td>
<td>3 ± 1.2 ± 1.2</td>
<td>83</td>
</tr>
</tbody>
</table>

Cultures were treated with the drug on day 3 and the nuclei stained and counted on day 6. Values are stated ± S.E.

• Myotubes are extremely short in these cultures.

The addition of bupivacaine, lignocaine, mepivacaine, cocaine or pentobarbital markedly inhibited myotube formation: the proportion of multinucleate cells was low and the few myotubes formed were very short, with generally less than 10 nuclei per myotube (Table 1), usually abnormally arranged and many of the myotubes were
Fig. 1. Phase-contrast micrographs of day-8 myotubes after treatment with 1-2 mM bupivacaine. A, 2 min; B, 7 min; and C, 50 min after treatment. The culture was treated on day 3 with $10^{-5}$ M cytosine arabinoside. Note disappearance of myotube on left-hand side in C and progressive rounding up of myotube on right-hand side in B and C. × 320.
dead. The addition of 1-2 mM bupivacaine resulted in all the cells becoming detached from the substrate. After removal of bupivacaine (0-6 mM) lignocaine or cocaine (1 or 2 mM), and pentobarbital (1 mM) from the cultures the number of myotubes present on day 8 approached that in control cultures. However, recovery following treatment with 5 mM lignocaine or cocaine, or 1 and 2 mM mepivacaine was negligible.

Barbitone (1 or 2 mM) hardly inhibited fusion; however, at these concentrations the nuclei were not as well lined up in the myotubes as in control cultures. Higher concentrations (5 or 10 mM) inhibited fusion by 79 and 100%, respectively. These concentrations also caused a reduction in the cell number, at least part of which was due to cell death, as shown by trypan blue staining.

**Effects of anaesthetics on fusion: effects of bupivacaine**

We studied the effect of bupivacaine more closely than that of the other anaesthetics. To minimize the effects of bupivacaine on cell division cultures were treated on day 4, just before fusion would normally have occurred. The percentage fusion was greatly diminished, 0-6 mM bupivacaine causing 60% reduction while 0-9 mM caused 90% reduction.

To determine how rapidly the bupivacaine effect could be reversed, confluent 3-day cultures were treated with 0-9 mM bupivacaine. On day 6, when no fusion had occurred, the bupivacaine was removed. Within 5 h several myotubes had formed and by day 8, the number of myotubes was close to that in control cultures. If the bupivacaine was not removed until day 7, few myotubes subsequently formed. Increasing the concentration of Ca$^{2+}$ by up to 20 mM, had little effect on the ability of bupivacaine to inhibit fusion.

**Effects of anaesthetics on myotube breakdown**

In addition to effects on fusion, nerve-blocking agents also made formed myotubes in culture disappear (Fig. 1). Bupivacaine, lignocaine, mepivacaine, cocaine and pentobarbital all caused myotubes to round up and fragment forming structures similar to the ‘myosacs’ (Fig. 1) which appear after treatment of myotubes with colchicine (Bischoff & Holtzer, 1968) (Table 2). The myoblasts and fibroblasts did not round up at concentrations which caused fragmentation of myotubes, and stayed attached to the culture dish.

The effect of bupivacaine was studied most closely. Experiments were usually performed on 7- and 8-day cultures. Bupivacaine disrupted myotube structure at a minimum concentration of 0-8 mM; in older cultures higher concentrations were needed. The first morphological changes were seen between 30 and 60 min; these included the appearance of bulges, myotube elongation and breakage. One hour after treatment, the number of myotubes per field of view had decreased to about 30% of the original number and those remaining were very short; 120 min after treatment, there were no myotubes (see Fig. 2, Table 3). The ‘myosacs’ remaining on the substrate following bupivacaine treatment were, like the myotubes and postmitotic myoblasts in control cultures, more heavily stained after May–Grunwald and Giemsa staining than other cells. A few of the products of myotube breakdown, the ‘myosacs’
became detached from the substrate, but counts of the total number of nuclei per field before and after drug treatment indicate that few myotubes detach from the plate during the course of fragmentation. Table 3 shows a time course of the percentage nuclei present in myotubes and the number of myotubes after treatment with 1-2 mM bupivacaine. Of the myosacs remaining on the substrate at 60 min (200 counted) 2% had 0 nuclei, 39% had 1 nucleus, 18% had 2, 6% had 3, and the remaining 13% had between 3 and 20 nuclei. If the medium was replaced within an hour of treatment normal-looking myotubes were present within 21 h and by 2 days after treatment there was extensive myotube formation in the treated cultures (Table 4).

Table 2. Nerve-blocking agents which cause the fragmentation of myotubes

<table>
<thead>
<tr>
<th>Drug</th>
<th>Minimum concentration required for myotube breakdown, mM</th>
<th>Time required for loss of majority of myotubes, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bupivacaine</td>
<td>0-8</td>
<td>1</td>
</tr>
<tr>
<td>Lignocaine</td>
<td>2-0</td>
<td>1</td>
</tr>
<tr>
<td>Cocaine</td>
<td>2-0</td>
<td>1</td>
</tr>
<tr>
<td>Mepivacaine</td>
<td>5-0</td>
<td>24</td>
</tr>
<tr>
<td>Pentobarbital</td>
<td>2-0</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3. Time course of myotube breakdown induced by 1-2 mM bupivacaine

<table>
<thead>
<tr>
<th>Time after treatment, min</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myotubes per field of view (X 10)</td>
<td>19-5±1.0</td>
<td>18±0.3</td>
<td>18±0.3</td>
<td>0-7±0.2</td>
<td>0-3±0.1</td>
</tr>
<tr>
<td>Total nuclei per field of view (X 40)</td>
<td>95±3</td>
<td>91±3</td>
<td>84±3</td>
<td>90±3</td>
<td>103±2</td>
</tr>
<tr>
<td>% nuclei in myotubes</td>
<td>13±2</td>
<td>5±2</td>
<td>5±2</td>
<td>2±1</td>
<td>3±1</td>
</tr>
</tbody>
</table>

Values are stated ± S.E.

Table 4. Regeneration of myotubes after removal of bupivacaine

<table>
<thead>
<tr>
<th>% nuclei in myotubes</th>
<th>Difference in % nuclei in myotubes (Day 8-Day 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6</td>
<td>Day 8</td>
</tr>
<tr>
<td>Control</td>
<td>25.1±0.2</td>
</tr>
<tr>
<td>Bupivacaine-treated</td>
<td>3.4±2.1</td>
</tr>
</tbody>
</table>

Cultures treated with 1-2 mM bupivacaine for 1 h on day 6. Drug and control cultures replaced with fresh medium. Duplicate control and bupivacaine-treated cultures were fixed, stained and counted on day 6 and day 8. Values are stated ± S.E.

Both 2 mM lignocaine and 2 mM cocaine disrupted the structure of myotubes in a similar manner to that seen with 1 mM bupivacaine. Within 2 days after removal of the drug, the number of myotubes approached that found in control cultures. Recovery following treatment with cocaine was less than that seen with lignocaine.
During the first 2 days after treatment, 1 mM lignocaine had no effect on myotube structure but after 3 days the myotubes were very short with large bulges surrounded by prominent thickenings. This effect was also observed following treatment over prolonged periods with low concentrations of bupivacaine. Treatment with 6 mM bupivacaine, or 10 mM lignocaine, mepivacaine and cocaine resulted in detachment of all the cells from the substrate.

A minimum temperature of 32 °C was required for the breakdown of myotubes using bupivacaine. However, myotubes in cultures treated on day four with 10⁻⁶ M cytosine arabinoside, which kills dividing cells, underwent bupivacaine-induced breakdown at 22 °C. These myotubes were more elongated than in untreated cultures.
The medium requirement for the effect of bupivacaine was surprisingly specific. Bupivacaine-induced breakdown occurred in DMEM, with or without Ca\(^{2+}\), Mg\(^{2+}\), CEE or FCS. However, it did not occur in MEM/HEPES, PBS or L15. Breakdown occurred in a mixture of 20% DMEM and 80% PBS. Increasing the calcium concentration by 5–20 mM significantly decreased the activity of the anaesthetics. The addition of 20 mM Ca\(^{2+}\) to the DMEM almost completely inhibited the action of the anaesthetic on myotube breakdown. Additional magnesium had no effect. Pentobarbital-induced breakdown was not affected by the addition of up to 20 mM Ca\(^{2+}\) to the DMEM. The Ca\(^{2+}\) ionophore, A23187, at concentrations of 5, 10 or 100 mM in the presence of 2 mM Ca\(^{2+}\) had no effect on the action of bupivacaine.

**DISCUSSION**

We have shown that local anaesthetics can inhibit fusion of myoblasts to myotubes, and can also cause bulging, elongation and breakdown of myotubes in rat newborn skeletal muscle cultures. These drugs inhibit fusion in a wide variety of systems; these include the fusion of secretory granules with the plasma membrane in exocytotic secretion in leukocytes (Goldstein, Lind, Hoffstein & Weissman, 1977), sperm (Collins & Epel, 1977), and mast cells (Kazimierczak, Peret & Maslinski, 1976) and the pathologically induced fusion of monocytes to giant cells in vivo (Papadimitriou & Sforcina, 1975) and Sendai virus-induced fusion (Post & Reeve, 1972). The mechanism of inhibition is unknown.

In our system, inhibition is unlikely to be a consequence of effects on cell division since low concentrations of lignocaine, cocaine and pentobarbital which have no significant effect on cell division, inhibit fusion. In addition, fusion is prevented when bupivacaine is added only 6 h prior to fusion to minimize effects on cell division. Fusion proceeds quite rapidly on removal of the drug. Local anaesthetics decrease cell motility (Gail & Boone, 1972) and inhibit membrane fusion directly (Poste & Reeve, 1972) so the fact that we do not observe synchronous fusion on removal of the drugs suggests that both these processes may be affected.

At intermediate concentrations all anaesthetics cause inhibition of both cell division and fusion. At high concentration all cells detach from the substrate which agrees with previous reports that high concentrations of local anaesthetics cause rounding up and detachment of 3T3, SV3T2 and BHK cells (Rabinovitch & De Stefano, 1974; Poste, Papahadjopoulos & Nicolson, 1975; Nicolson, Smith & Poste, 1976).

The breakdown of myotubes seen in our cultures parallels the degeneration of mature myotubes seen in vivo after some local anaesthetics are injected into skeletal muscle and shows that the myotoxic effect is due to a direct interaction of the anaesthetic with the muscle fibres since fibroblasts and myoblasts in culture do not round up or detach under the conditions used for myotube and skeletal muscle breakdown. In addition the simplest interpretation of the experiment in which myotubes were reformed after removal of bupivacaine from fused cultures (Table 4) is that some regeneration of myotubes from the fragmented ‘myosacs’ has occurred. The number of myotubes in bupivacaine-treated cultures on day 8 is significantly greater than would be expected if the increase in myotubes was due solely to recruitment of postmitotic
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myoblasts, which suggests that some of the regeneration seen in vivo may arise directly from the damaged fibres.

The rounding up of the cell membrane seen in macrophages and polymorphonuclear leukocytes after treatment with anaesthetics (Rabinovitch & DeStefano, 1974 b, 1976; Goldstein et al. 1977) which does not seem to be directly related to disruption of microfilaments and microtubules seen in 3T3 cells treated with local anaesthetics (Nicolson, Smith & Poste, 1976) may well be important in myotube breakdown. Colchicine causes myotube breakdown into large myosacs over a period of days (Bischoff & Holtzer, 1968, and unpublished observations) and cytochalasin D causes extensive elongation without breakdown (Miranda & Godman, 1973) while a combination of cytochalasin B and colcemid causes rounding up of myotubes, myoblasts and fibroblasts, with shedding of cytoplasmic droplets from the mononucleated cells and condensation of the 10-nm filaments (Croop & Holtzer, 1976; Bennett et al. 1978). Anaesthetics cause rapid elongation and extensive fragmentation of myotubes, but seem to have few effects on the morphology of other cell types in the culture so effects on microfilaments and microtubules alone would seem insufficient to explain the results.

Local anaesthetics have other effects on cell membranes (Seeman, 1972), which may be important in myoblast fusion and myotube breakdown. Cell aggregation and ligand-induced redistribution of membrane surface receptors are affected in a way which can be mimicked by a combination of colchicine and cytochalasin B (Ryan, Unanue & Karnovsky, 1974; Poste et al. 1975). Membrane surface area and fluidity are increased and so is the resistance of erythrocytes to haemolysis (Seeman, 1972; Sheetz & Singer, 1974). Ca²⁺ bound to phospholipids in the membrane is displaced by cationic anaesthetics and increased by barbiturates (Blaustein & Goldman, 1966; Papahadjopoulos, 1970).

We found no direct differences between the actions of the barbiturate pentobarbital and those of the cationic anaesthetics bupivacaine, lignocaine and cocaine on either inhibition of myoblast fusion or myotube breakdown. Increasing the Ca²⁺ concentration had no effect on bupivacaine-induced inhibition of fusion. However, it reversed the effect of bupivacaine but not pentobarbital on myotube breakdown. During histamine release from mast cells (Kazimierczak et al. 1976) and the acrosome reaction of sea-urchin sperm (Collins & Epel, 1977) when fusion occurs between an intracellular membrane and the plasma membrane, raising the external Ca²⁺ concentration overcomes the inhibition of fusion induced by cationic anaesthetics. In these cases and also in myotube breakdown the extra Ca²⁺ probably acts by decreasing the membrane binding of cationic anaesthetics to below the threshold concentration necessary for action. It is surprising that increased Ca²⁺ cannot overcome the bupivacaine-induced inhibition of myoblast fusion. Increased Ca²⁺ promotes the binding of pentobarbital to membrane phospholipids and has no effect on the action of this drug on either fusion or myotube breakdown.

If myotube breakdown were related to a decrease in the calcium flux across the membrane and a subsequent drop in intracellular Ca²⁺ levels the divalent ionophore A23187, which increases the calcium flux across the myotube membrane (Schudt &
Pette, 1975) should override drug action. No such effect was seen. In chick muscle cultures where myoblast fusion is inhibited by suboptimal calcium levels in the medium the same ionophore also fails to promote fusion (Schudt & Pette, 1975). These results suggest that in both inhibition of fusion and myotube breakdown the calcium flux is not a primary factor.

The responses of different cell types to local anaesthetics are very varied, probably reflecting the fact that these drugs can affect several important cellular processes. Until more is known about the molecular mechanism of interaction and the anaesthetics in each of these processes it will be difficult to pinpoint which effect is most important in any particular case.

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