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Diazepam Inhibits Myoblast Fusion and Expression of Muscle Specific Protein Synthesis

Abstract. *The presence of diazepam in cultures of chicken embryo myoblasts arrests normal muscle cell differentiation. High concentrations of the drug reversibly prevent myoblasts from fusing to form multinucleated myotubes. Lower concentrations of diazepam allow cell fusion to occur, but inhibit the synthesis and accumulation of myosin heavy chain, implying that cell fusion does not obligate myoblasts to synthesize and accumulate large quantities of muscle specific protein. The effect of diazepam on muscle cells in culture is direct and specific.*

Diazepam is a widely used muscle relaxant (*1*), although its site and mechanism of action on a molecular level is unknown. Recent studies have demonstrated a specific receptor in the central nervous system for benzodiazepines, a group of compounds which includes diazepam (*2*). It is not known whether the muscle relaxant effects of diazepam are central or peripheral. In some studies specific receptors for diazepam have not been found in adult skeletal muscle (*2*); in other studies, direct effects on skeletal and cardiac muscle were observed (*3*).

An attempt to elucidate the direct biochemical effects of the drug on embryonic skeletal muscle cells was made by adding diazepam (Valium, Roche) to the culture medium of chick myogenic cells undergoing differentiation. Although polyethylene glycol and ethanol were used to solubilize the diazepam for stock solutions, resulting in final concentrations of 0.2 percent polyethylene glycol and 0.05 percent ethanol in the culture medium, no effects on the cultures were observed by the presence of the vehicle without diazepam.

Cells isolated from breast muscle of 12-day-old chick embryos align with each other and fuse to form multinucleated myotubes (*4*). After cell fusion, there is a large increase in both the synthesis and accumulation of muscle specific proteins, of which the myosin heavy chain is a major component (*5*, *5a*). After 5 days in culture, myotubes are normally striated and undergo spontaneous contractions (*6*). Normal chick myogenic cultures at days 2, 3, and 5 (Fig. 1, A to C) were compared with similar cultures grown in the presence of 100 μM diazepam (Fig. 1, D to F) (*7*). At day 2 the alignment of the diazepam-treated cells was similar to that of the control cultures, but normal fusion was inhibited. By day 5, few multinucleated myotubes were present in the treated cultures, although the close proximity of aligned myoblasts made it difficult to measure the exact extent of fusion. Although a lower concentration of diazepam (50 μM) did not inhibit cell fusion, myotubes did not enlarge, become

Table 1. Synthesis and accumulation of total protein and myosin heavy chain in control and diazepam-treated cultures. Total protein content was measured by the method of Lowry (*8*). Incorporation of [^{3}H]leucine into total protein was determined by precipitating samples in trichloroacetic acid, and measuring the radioactivity collected on glass fiber filters. The incorporation of [^{3}H]leucine into myosin heavy chains and the determination of myosin heavy chain content have been described (*9*). The results represent the data from one of three replicate experiments. While the actual numbers varied from experiment to experiment (due to variations in the number of cells per culture), the magnitude of the inhibition of myosin heavy chain synthesis and accumulation were similar in each case. At the termination of each experiment control cultures contained 5.97 ± 2.02 times more myosin heavy chain and incorporated 5.53 ± 2.12 times more leucine into myosin heavy chain than diazepam-treated cultures. In contrast, control cultures contained 1.57 ± 0.42 times the total protein as diazepam-treated cultures, and incorporated 1.43 ± 0.25 times the leucine into total protein. Results given are per culture.

Days in culture	Incorporation of [^{3}H]leucine				Total protein (mg)		Myosin heavy chain (μg)	
	Total protein (10^5 count/min)		Myosin heavy chain (10^3 count/min)					
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
2	1.27	0.90	0.25	0.09	0.81	0.80	1.20	1.00
3	2.40	1.66	1.64	0.32	1.54	1.11	9.80	4.80
4	4.35	2.84	4.43	1.41	1.54	1.01	21.60	7.80
5	5.17	4.41	12.70	2.30	1.98	1.57	28.40	6.00

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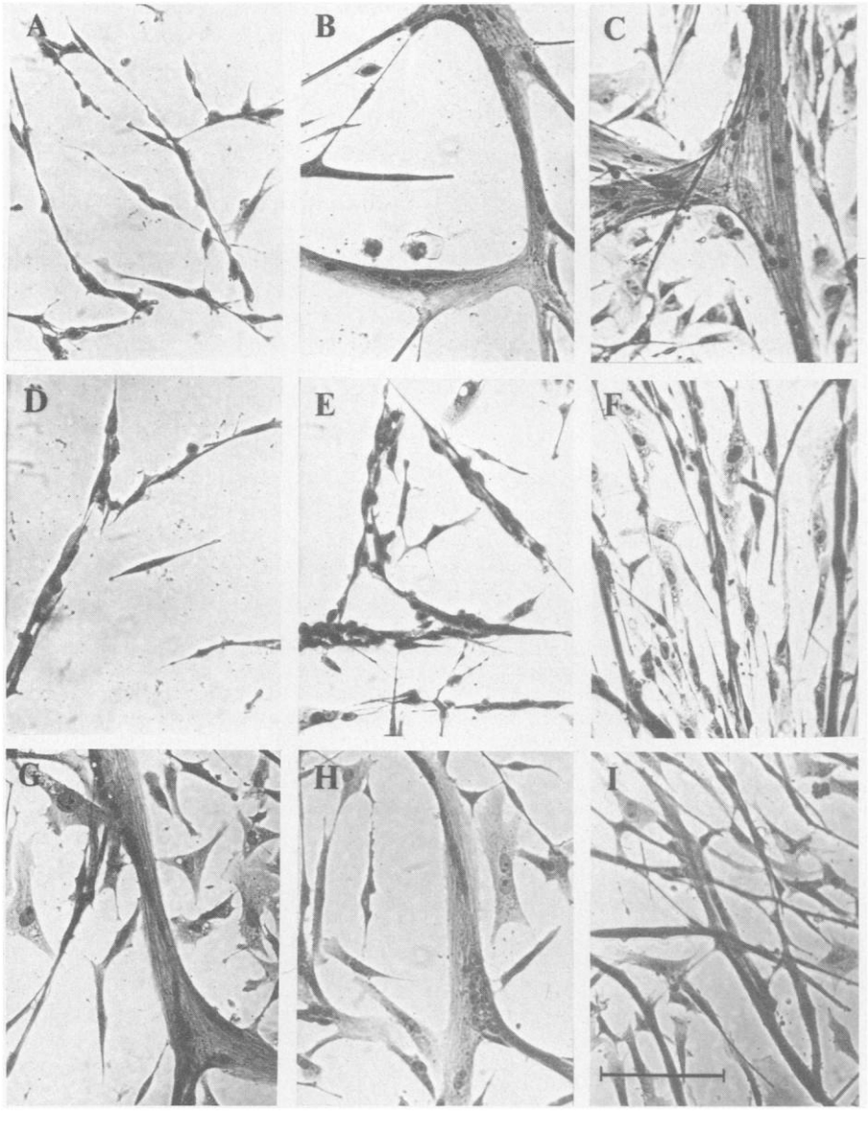
striated (Fig. 1, H and I), or develop spontaneous contractions.

The inhibition of fusion by diazepam is reversible. Cultures grown for 3 days in the presence of 100 μM diazepam were replenished with fresh medium lacking the drug; within 24 hours, extensive normal cell fusion had occurred (Fig. 1G).

Cell fusion normally precedes the synthesis and accumulation of muscle specific proteins; however, myosin synthesis and accumulation can occur in the absence of cell fusion (*8–10*). We investigated the question of whether diazepam-treated cultures synthesized and accumulated myosin heavy chain. Incorporation of leucine into total protein was inhibited as much as 30 percent (Table 1), but leucine incorporation into myosin heavy chain was more severely inhibited. A decrease in the accumulation of myosin heavy chain occured as well. Diazepam-treated cultures contained 75 to 90 percent of the total protein found in control cultures, but the content of myosin heavy chain was only 15 to 25 percent that of untreated cultures. Thus 100 μM diazepam not only inhibited myoblast fusion, but also the synthesis and accumulation of muscle specific protein. Diazepam had relatively little effect on nonmuscle cells. The cultures contained some fibroblasts that continued to divide in the presence of diazepam; they eventually overgrew the unfused myoblasts that had ceased to divide (*11*).

The inhibition of fusion, myosin heavy chain synthesis, and accumulation caused by diazepam is dose related. Cultures were prepared at various concentrations of the drug and grown for 5 days. As little as 10 μM diazepam inhibited myosin heavy chain synthesis relative to that in control cultures, although it had a lesser effect on total protein synthesis (Fig. 2). Similar results are shown for the accumulation of myosin heavy chain. Maximum inhibition of myosin heavy chain synthesis was achieved at 50 μM diazepam, even though cell fusion occurred at this concentration (Fig. 1, H and I). Thus it appears that myoblast cell fusion does not obligate the cell to synthesize the large quantity of muscle specific protein characteristic of normal muscle development.

The mechanism by which diazepam inhibits muscle differentiation is unknown. Balzer *et al.* (*12*) found evidence for the inhibition of calcium transport in the presence of benzodiazepines. Calcium is a requirement for myoblast fusion (*5a, 13*), and it is possible that diazepam restricts the availability of calcium to the cell. However, removal of calcium from the culture medium inhibits fusion more strongly that it inhibits myosin synthesis (*5a, 8, 10, 13*), while the addition of diazepam affects myosin synthesis more



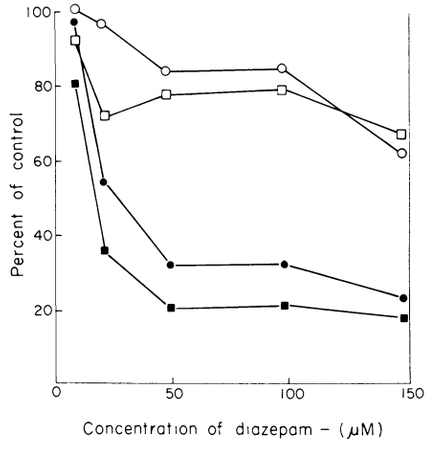


Fig. 1 (left). Effect of diazepam on myoblast fusion and maturation of myotubes. (A) Normal chick myogenic culture at 2 days, (B) 3 days, and (C) 5 days. (D) Cells grown in 100 μM diazepam for 2 days, (E) 3 days, and (F) 5 days; (G) cells grown in 100 μM diazepam for 3 days, and then for 1 day more in fresh medium lacking diazepam; (H) cells grown in 50 μM diazepam for 3 days, and (I) 5 days. Photographs are of fixed, stained cultures. Scale bar, 0.1 mm. Fig. 2 (above). Effect of diazepam on myosin heavy chain synthesis and accumulation. Cultures grown for 5 days in various concentrations of diazepam were labeled for 1 hour with [^{3}H]leucine and assayed for (○) total protein synthesis, (□) total protein content, (●) myosin heavy chain synthesis, and (■) myosin heavy chain content as described in the legend to Table 1. Data are expressed as percentages of untreated cultures.

strongly than it does fusion. Diazepam will be important in evaluating mechanisms of myoblast fusion and myosin heavy chain synthesis, as well as in determining the relationship between contractility and myosin heavy chain synthesis.

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Pollen Abortion in T Cytoplasmic Male-Sterile Corn (*Zea mays*): A Suggested Mechanism

Abstract. *A rapid replication of mitochondria (20- to 40-fold increase) occurs between the precallose and tetrad stages in the tapetum of N and T corn* (Zea mays) *anthers, followed by mitochondrial, tapetal, and pollen breakdown in T anthers. It is suggested that the altered DNA in T mitochondria may malfunction under these stress conditions.*

With appropriate fixation and staining procedures, DNA fibrils can be demonstrated in plastids and mitochondria of higher plants. These fibrils form the basis of a limited nucleic acid system in these organelles, over and above the elaborate system found in the nucleus. Techniques such as the one described here (*1*) clearly show the DNA fibrils in both mitochondria and proplastids (Fig. 1, arrows).

Although limited in quantity, organellar DNA has the essential properties of nuclear DNA in being made up of linearly arranged base pairs and in having the capacities of self-replication and RNA transcription. It has become evident that this extranuclear DNA provides the basis of those characters that are not inherited in a Mendelian manner, and which for this reason have been termed "cytoplasmic."

The T-type male sterility in corn (*Zea mays*) is an example of cytoplasmic inheritance [reviewed in (*2*)]. It is not transmitted through the male, and crosses and backcrosses between male-sterile plants (T) and fertile maintainer plants (N) do not segregate. In a classic early work, Rhoades (*3*) showed that replacement of all nuclear chromosomes in a male-sterile line with chromosomes known to be free of sterility factors had no effect on the degree of sterility.

More recent work has shown that mitochondria from N and T plants react differently to toxins of *Helminthosporium maydis* Nisikado and Miyake, the agent of southern corn leaf blight (*4*). Levings and Pring (*5*) have shown that mitochondrial DNA's from N and T plants differ significantly after treatment with restriction endonucleases. We have observed that mitochondria in the tapetum and middle layers of plants with T cytoplasm become disorganized internally shortly after meiosis (*6*), and that this results in early tapetal vacuolation and degeneration. Since the tapetum serves as the source of nutrients for the young microspores, its early degeneration may well be related to pollen abortion. The T type of sterility in corn is thus clearly inherited cytoplasmically and would appear to be conditioned by an altered DNA in the mitochondria of plants with T cytoplasm.

Recent studies (*7*) on mitochondrial size and number in inbred lines of F_{44} N and T corn plants suggest an explanation for the mitochondrial breakdown observed in anthers with T cytoplasm. A statistically significant increase in numbers and decrease in size of mitochondria occurs in certain anther cells of both N and T plants (Table 1), but mitochondrial degeneration and microspore abortion occur only in plants with T cytoplasm. This mitochondrial replication is rapid (largely between precallose and tetrad stages, a period of some 3 days) and immediately precedes the observed degeneration in anthers with T cytoplasm.

During this period, sporogenous cells show a fourfold increase in number (as a result of the meiotic divisions), tapetal cells show a 1.3-fold average increase in number (as the result of occasional mitotic divisions), and the cells in both tissues show a four- to sixfold increase in volume. While these moderate increases are taking place in cell dimensions, mitochondrial numbers increase almost 20 times in sporogenous tissue and some 40 times in the tapetum, and individual mitochondrial volumes decrease 12 to 15 times (Table 1, ratio S_4/S_1). These changes are observed in the tapetum and sporogenous cells of both fertile and sterile anthers, but not in other anther tissues.

Other workers (*8*) have measured and counted mitochondria in plant cells, but we believe this is the first time that fertile and cytoplasmic male-sterile plants have been compared by such measurements. Also, the decrease in mitochondrial size and the rapid rate of mitochondrial replication observed in the present study, especially in the tapetum, appears to be unusual.

If organelles contain multiple and possibly a variable number of copies of the genome, as seems likely (*9*), we may question the effect of this rapid increase in number and decrease in size on mitochondrial DNA and protein mechanisms: This might well constitute a condition of

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