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BIOCHEMICAL AND MORPHOLOGICAL EFFECTS OF 20,25-DIAZACHOLESTEROL ON CULTURED MUSCLE CELLS

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Abstract—Effects of 20,25-diazacholesterol (DAC), a myotonia-inducing drug, were evaluated on certain biochemical and morphological properties of embryonic rat muscle cells grown in tissue culture. During DAC treatment, muscle fibers exhibited spontaneous contractions that changed from coarse twitches to finer fibrillation movements. The ultrastructural alterations produced by DAC were smeared Z-lines, disorganized myofibrils, occasional honeycomb appearance of membranes and large vacuoles connected to zipper-like structures. Biochemically, a microsomal fraction prepared from DAC-treated cells (compared to that of normal cells) showed a 30–45 per cent decrease in the isoproterenol-enhanced and the NaF-enhanced adenylate cyclase activity. However, the β-adrenergic receptors, through which isoproterenol activates the enzyme, showed no change in density or affinity as judged by the binding of [^{125}I]iodohydroxybenzylpindolol. That indicated that DAC treatment caused an uncoupling of β-receptor–adenylate cyclase interaction. Guanylate cyclase and cyclic GMP-phosphodiesterase were both markedly increased in DAC-treated cells, indicating a greater turnover of cyclic GMP. Binding of [^{3}H]concanavalin A to DAC-treated muscle membranes was decreased 20–40 per cent. The data indicate that DAC exerts a direct influence on muscle fibers, affecting their functional, biochemical and morphological properties.

The myotonic phenomenon (myotonia) is characterized by the failure of skeletal muscle to relax properly after voluntary exercise or after electrical or mechanical stimulation. This is a heritable disorder in humans and in a certain strain of goat. The etiology and pathophysiology of myotonia are not yet fully understood. Myotonia, similar in many respects to that found in human and goat, can also be induced in experimental animals such as the rat by treating them with 20,25-diazacholesterol (DAC) or with aromatic monocarboxylic acids [1]. 20,25-DAC is an anticholesterolemic agent which prevents the reduction of desmosterol (a precursor of cholesterol) to cholesterol [2]. 20,25-DAC-induced myotonia provides an important animal model [3]. However, the effect of the drug on the whole animal appears to be complex. Besides muscle, the drug also affects the metabolism and physiology of other tissues [4–6]. To study the direct influence of DAC on muscle tissue, rat skeletal muscle has been grown in culture in the presence of DAC. We report certain morphological and biochemical effects of DAC on cultured embryonic rat skeletal muscle.

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MATERIALS AND METHODS

Cell culture and 20,25-DAC treatment. Rat muscle cultures were established from the gastrocnemius muscle of 19-day-old embryos as described previously [7]. The muscle cells were grown at a concentration of 50,000 cells/ml of medium in 100 mm and 35 mm petri dishes for biochemical and ultrastructural studies respectively.

Cultures were examined daily under phase-contrast inverted-microscopy. When most of the myotubes seemed to be well cross-striated and vigorously contracting (usually at 7 or 8 days of growth), treatment with DAC was initiated. Pulses of 0.05 mM DAC dissolved in complete culture medium were given for 15–20 min for 3 consecutive days. The cultures were then harvested for the biochemical studies and processed for the electronmicroscopic studies as previously described [7–9]. Concentrations of DAC greater than 0.05 mM and exposures greater than 20 min per day were found to be toxic to the cells. For ultrastructural cytochemistry, some cultures were fixed with tannic acid for the visualization of surface membranes and sarcotubular structures [10] and others were stained with peroxidase-diaminobenzidine reaction for visualization of conconavalin A binding sites [11]. Ten separate experiments were performed; cultures untreated with DAC served as controls in every experiment.

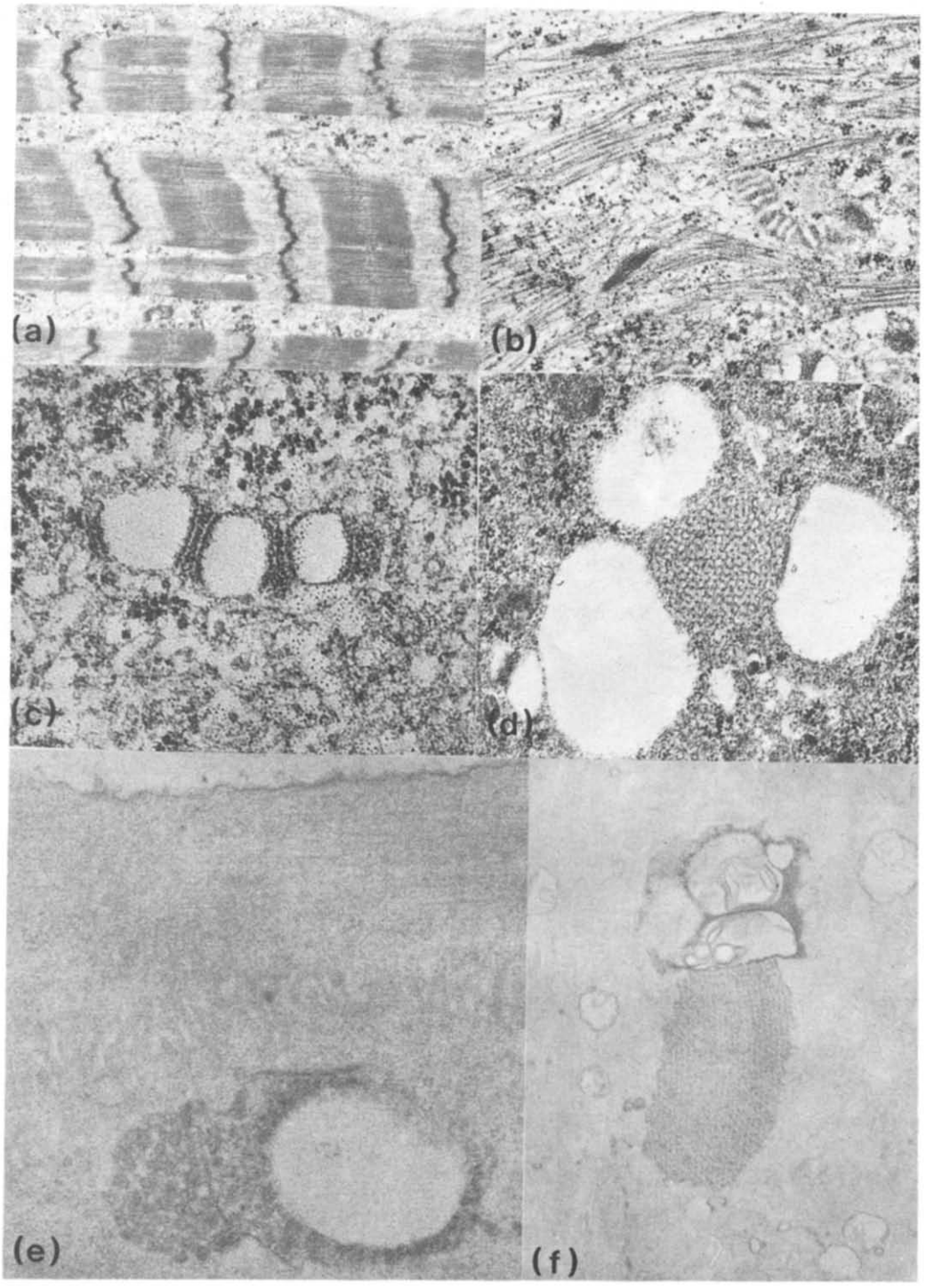

Fig. 1. Ultrastructural features of control and 20,25-diazacholesterol-treated cultured rat skeletal muscle: (a) control cultured muscle × 10,500 (panels b, c, d, e and f depict 20,25-DAC-treated cultured muscle); (b) streaming of Z-bands and disorganization of myofibrils × 24,600; (c) vacuoles connected with "zipper"-like structures × 79,200; (d) honeycomb appearance of sarcoplasmic reticulum between three large vacuoles × 33,000; and (e) and (f) tannic acid staining of the plasmalemma vacuoles and honeycomb structures (e, × 41,400 and f, × 15,800).

Biochemical experiments. For biochemical studies the cells were harvested in 0.25 M sucrose and 10 mM N^2-hydroxyethylpiperizine-N^2-ethanesulfonic acid (HEPES) (pH 7.4) and centrifuged at 1000 *g* for 10 min. All procedures were carried out at 4°. The pellet was washed twice with the same medium; then it was weighed and homogenized in 8 vol. of 10 mM HEPES (pH 7.4) with a polytron PT 10 homogenizer at top speed for 15 sec. The homogenate was centrifuged at 1000 *g* for 5 min and the sediment was discarded. The supernatant fraction was centrifuged at 100,000 *g* for 30 min. The pellet was resuspended in 0.6 M KCl, 10 mM HEPES (pH 7.4) and allowed to stand for 20 min. The suspension was centrifuged at 100,000 *g* for 30 min. The resulting pellet was washed with excess 10 mM HEPES (pH 7.4) and used as a source of "membranes" for biochemical assays. This fraction contains the membranes of plasmalemma, sarcoplasmic reticulum and mitochondria.

Adenylate cyclase [12], cAMP and cGMP phosphodiesterase [13], guanylate cyclase [14], and β-adrenergic receptor binding using [^{125}I]-iodohydroxybenzylpindolol (IHYP) [15] were assayed by previously published methods.

Concanavalin A (Con A) binding was determined by the microfuge centrifugation technique. The incubation medium in a final volume of 100 μl contained 100 μg membrane protein, 0.1% bovine serum albumin, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 μM [^{3}H]Con A and 50 mM Tris–HCl (pH 7.5). Parallel assay tubes contained 0.2 M α-methyl-D-mannoside to determine the binding of [^{3}H]Con A to non-mannoside sites. The difference in [^{3}H]Con A binding between the absence and presence of α-methyl-D-mannoside was taken as the amount of [^{3}H]Con A specifically bound to mannoside sites. The incubation was for 20 min at 37°. At the end of the incubation, 200 μl of cold (2°) 50 mM Tris–HCl, (pH 7.5) was added and the tubes were centrifuged immediately in a Beckman model 162 microcentrifuge. The resulting pellet was washed twice with 300 μl of the same cold buffer. No radioactivity was detected in the final wash, indicating complete removal of unbound [^{3}H]Con A. Finally, the tip of the microfuge tube containing the pellet was cut out into a glass scintillation vial. To the vials was added 0.5 ml of 2% sodium dodecylsulfate, and the vials were shaken for 12–24 hr to solubilize the membrane pellet. The vials were then counted with 10 ml Aquasol (New England Nuclear Corp., Boston, MA) in a Packard model 2660 spectrometer with a ^{3}H counting efficiency of 40 per cent.

Protein content was estimated by the method of Lowry *et al.* [16]. In some experiments where whole homogenate was used, the results were expressed per mg of non-collagenous protein which was extracted [17] and estimated as above.

RESULTS

Morphological. After 15 min of DAC treatment, spontaneous contractions of the muscle fibers became quite irregular and fibrillation-like. The change in contraction pattern persisted for 3–4 hr following treatment, after which the normal contraction pattern returned. After 15 min of DAC treatment no morphological abnormalities would be observed with light microscopy. Longer treatment, however, resulted in a distinct granular appearance of the cultured muscle fibers in our preliminary studies. Therefore, the present studies were performed on the cultures that showed definite change in the contraction pattern but before the appearance of granularity in order to avoid non-specific changes due to possible toxicity of DAC. For morphological studies, cells were harvested 3 hr after DAC treatment, when contractile properties had returned to normal.

Several ultrastructural abnormalities were evident in the DAC-treated muscle cultures when compared to cultured normal muscle cells. The most prominent among them were irregularities and smearing of the Z-disks, disorganization of the myofibrils, prominent honeycomb appearance of the sarcoplasmic reticulum, and vacuoles (presumably of sarcoplasmic reticulum origin) connected to "zipper"-like structures (Fig. 1). Tannic acid staining revealed irregularities of the plasmalemma as well as proliferated sarcotubular structures in the DAC-treated cultured muscle (Fig. 1). Staining of Con A binding to the membranes of DAC-treated muscle cultures appeared weaker than that of control muscle cultures (data not shown), and that was further illustrated by biochemical estimations (see below).

Biochemical. Previous physiological and biochemical studies in human and animal myotonic models, including the DAC model, indicated that several membrane-bound functions are altered in skeletal muscle [3, 18]. We therefore investigated the effect of DAC on some of the muscle cell membrane-bound functions in the present study. Adenylate cyclase is primarily localized in the plasmalemma of many eukaryotic cells, and the enzyme is regulated both *in vivo* and *in vitro* by several hormones [19]. The enzyme activity was detectable in isolated membranes of muscle cells after 11 days in culture, and it was enhanced by NaF and catecholamines such as isoproterenol (Table 1). GTP enhanced the basal activity and its effect in the presence of isoproterenol was additive (Table 1); GTP is known to enhance the response of adenylate cyclase to different hormones in several tissues [20]. The membranes derived from the cells treated with DAC showed no significant change in the basal adenylate cyclase activity, but the activity in the presence of NaF and isoproterenol was significantly lower than in control (Table 1). These results are in general agreement with our earlier *in vivo* studies on the plasmalemma isolated from the skeletal muscles of rats rendered myotonic with DAC treatment where also the adenylate cyclase stimulated by isoproterenol and NaF was found to be decreased [18].

Isoproterenol is known to enhance adenylate cyclase by interacting with the β-adrenergic receptor [19]. Decreased response of adenylate cyclase to isoproterenol in DAC-treated cells could be due to a change in the affinity of the enzyme for isoproterenol or due to a decreased number of β-receptor sites. To distinguish between these possibilities, we estimated the enzyme activity at several different concentrations of isoproterenol. Dose–response

Table 1. Effect of 20,25-DAC (0.05 mM) treatment on adenylate cyclase (AC), cAMP-phosphodiesterase (PDE) and β-adrenergic receptor (βAR) binding of cultured rat muscle cells*

	Adenylate cyclase						
	Basal	NaF (10 mM)	GTP (10 μM)	ISOP (10 μM)	ISOP + GTP	cAMP-PDE	βAR Binding
Control	119 ± 16	1635 ± 140	180 ± 23	243 ± 20	338 ± 30	16.3 ± 2.5	4.6 ± 0.8
DAC	102 ± 12	1034 ± 170	120 ± 25	144 ± 26	244 ± 22	17.5 ± 2.3	4.2 ± 0.4
P†	NS‡	< 0.01	< 0.05	< 0.01	< 0.05	NS	NS

* Values are means of four assays ± S.D. Units: AC, pmoles cAMP formed·(mg protein)$^{-1}$·10 min^{-1}; PDE, pmoles cAMP hydrolyzed·(mg protein)$^{-1}$·min^{-1}; and βAR, fmoles [^{125}I]IHYP bound/mg protein.

† P, Student's *t*-test.

‡ NS, not significant.

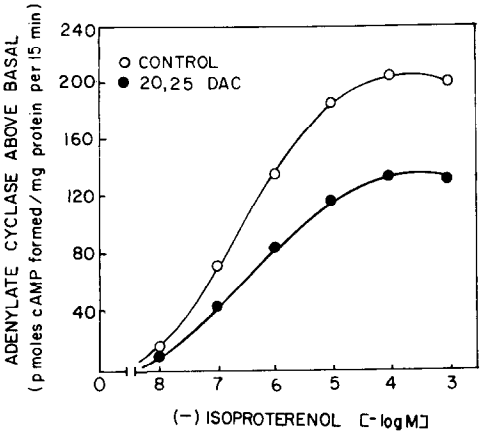

Fig. 2. Dose-response of adenylate cyclase to (−)-isoproterenol in control condition and after treatment with 20,25-DAC.

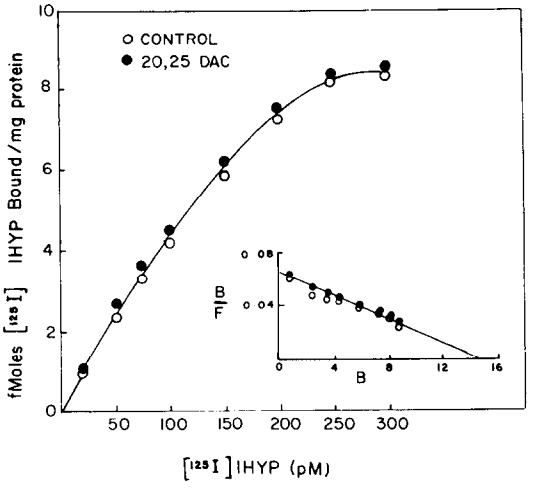

Fig. 3. Specific binding of [^{125}I]IHYP to control and 20,25-DAC-treated muscle cells as a function of increasing concentration of [^{125}I]IHYP. The insert shows the Scatchard plot.

curves (Fig. 2) of isoproterenol indicated that maximal activity in both control and DAC-treated cells occurred at a concentration of 1×10^{-5} M. The concentration of isoproterenol needed to cause half-maximal activation of adenylate cyclase was 4×10^{-7} M and was similar for both normal and DAC-treated cells. Thus, the affinity of the enzyme toward isoproterenol was not affected by DAC, but the extent of enzyme response was decreased at all concentrations of isoproterenol tested. The β-receptors were identified by following binding of [^{125}I]IHYP, a drug known to bind specifically to β-adrenergic receptors [15]. The binding was saturable both in control and DAC-treated cell membranes (Fig. 3). Scatchard [21] analysis of the saturation binding data (Fig. 3, inset) revealed that [^{125}I]IHYP bound to a single group of β-receptor sites with an apparent dissociation constant (K_d) of 80 pM. At apparent saturation, the receptor concentration was 15 fmoles of [^{125}I]IHYP bound per mg membrane protein in both normal and DAC-treated cells. Thus, DAC treatment neither altered the affinity of the adenylate cyclase towards isoproterenol nor did it alter the affinity or density of the β-adrenergic receptors sites in muscle cells.

The decreased level of adenylate cyclase activation by stimulants may reflect a decreased level of cyclic AMP (cAMP) production in DAC-treated cells. However, the cAMP level would also depend on the activity of phosphodiesterase (PDE) which is involved in the breakdown of cAMP (cAMP-PDE). cAMP-PDE is known to exist both in cytoplasmic and in particulate fractions [22]. We therefore estimated PDE in the whole cell homogenates and found it to be about the same in control and DAC-treated cells (Table 1).

Since the results of cyclic GMP (cGMP) action are, under certain conditions, opposite to those of cAMP [23], we estimated the activity of the enzymes involved in cGMP metabolism in control and DAC-treated cells. Guanylate cyclase catalyzes the synthesis of cGMP from GTP, and it is known to be localized both in membrane and cytosol fractions of many tissues [23], including skeletal muscle [24, 25]. Because of its dual localization, we determined the guanylate cyclase activity in whole cell homogenates. The basal activity (in the absence of added stimulant)

Table 2. Guanylate cyclase and cGMP-PDE of control and 20,25-DAC-treated cells*

	Guanylate cyclase		
	Basal	Triton	cGMP-PDE
Control	72 ± 5	433 ± 54	8.4 ± 1.2
DAC	112 ± 14	688 ± 62	15.6 ± 1.8
P†	< 0.05	< 0.01	< 0.01

* Values are means of four assays ± S.D. Units: guanylate cyclase, pmoles cGMP formed·(mg protein)$^{-1}$·10 min^{-1}; and PDE, pmoles cGMP hydrolyzed·(mg protein)$^{-1}$·min^{-1}.

† P, Student's *t*-test.

was about 60 per cent greater (expressed per mg non-collagenous protein) in the cells treated with DAC than in control cells (Table 2). Triton X-100 (0.5%), which is known to stimulate guanylate cyclase [23], enhanced the enzyme activity about 6-fold over the basal activity in both control and DAC-treated cells (Table 2). The Triton-stimulated activity was also greater in DAC-treated cells than in controls. The enhancement of guanylate cyclase in DAC-treated cells contrasts with the situation observed with adenylate cyclase (Table 1). Phosphodiesterase involved in cGMP hydrolysis (cGMP-PDE) was also found to be enhanced in DAC-treated cells (Table 2). Thus, higher levels of both guanylate cyclase and cGMP-PDE indicate greater turnover of cGMP in DAC-treated cells.

Concanavalin A (Con A) is known to bind to glycoprotein moieties in cell membranes, primarily to those containing mannoside and glucoside groups [26]. Our preliminary experiments with morphological staining for Con A indicated a decrease in Con A sites in DAC-treated muscle cells. To further confirm and quantitate this decrease, we estimated the binding of [^{3}H]Con A to isolated membranes of control and DAC-treated cells. In cultured muscle cells, Con A seems to bind predominantly to mannoside sites because more than 80 per cent of total [^{3}H]Con A binding was inhibited by α-methyl D-mannoside (Table 3). The non-mannoside binding sites of Con A were about 10 per cent of the total Con A binding sites. [^{3}H]Con A binding was more in younger (8 days old) cultures than in older ones (14 days old), which was the same for the control and DAC-treated cells. DAC treatment in the two age groups resulted in a loss of [^{3}H]Con A binding sites, the loss being more pronounced in older cultures than in younger ones (Table 3). Our present experiments do not clarify whether the decrease in Con A binding is due to loss of glycoprotein residues in the surface membrane or in other membranes because the binding studies were done with a crude particulate fraction which contains plasmalemma, sarcoplasmic reticulum and mitochondria.

Since previous studies on rats treated with DAC revealed close correlation between desmosterol accumulation and exogenous cholesterol [3], we determined cholesterol in growth medium used for rat muscle cultures and desmosterol in both the medium and muscle cultures by gas–liquid chromatography. The growth medium was found to contain 1.7 mg cholesterol/ml. Desmosterol was not detected in either the medium or the homogenates of cultures.

DISCUSSION

The myotonia induced by DAC in adult rats has been studied in considerable detail [3]. However, in those studies it was not very clear whether the changes in muscle properties were due to a direct effect of the drug on muscle fibers or were responses secondary to changes in other systems of the animal. We reasoned that evaluation of the direct effect of DAC can be made using rat skeletal muscle cells grown in culture, where the extraneous factors such as neural and humoral systems are not in operation. The data presented here indicate that primary cultures of rat muscle cells undergo morphological and biochemical changes when treated with low concentrations of DAC (0.05 mM) for a very short period (3 days, 15 min of exposure each day). To our knowledge this is the first detailed study on the direct effects of DAC on muscle cells in culture. The prominent morphological alterations induced by DAC in muscle cells were: smeared Z-disks, disorganized myofibrils, honeycomb appearance of sarcoplasmic

Table 3. Effect of 20,25-DAC (0.05 mM) treatment on [^{3}H]Con A binding of rat muscle cell membranes*

	Age of cultures					
	8 Days			14 Days		
[^{3}H]Con A binding	Control	DAC-treated	Decrease (%)	Control	DAC-treated	Decrease (%)
Total	1.8 ± 0.3	1.3 ± 0.2	28	1.07 ± 0.22	0.56 ± 0.08	48
Non-mannoside binding (α-Methyl-D-mannoside resistant)	0.18 ± 0.02	0.14 ± 0.02	23	0.13 ± 0.01	0.08 ± 0.008	39
Mannoside binding (α-Methyl-D-mannoside inhibited)	1.65 ± 0.25	1.2 ± 0.08	28	0.95 ± 0.15	0.50 ± 0.04	48

* Values, expressed as pmoles [^{3}H]Con A bound per mg protein, are means ± S.D. of three experiments each assayed in duplicate.

reticulum, proliferation of sarcotubular structures, and irregularities in the surface membrane including a decrease in Con A binding. Whether these morphological changes are interrelated or the drug affects each component separately is not clear from our experiments. Interestingly, these morphological changes closely resemble the structural changes previously reported for human myotonia as well as rat myotonia induced by DAC [27]. Thus, cultured muscle fibers provide another avenue of investigating the influence of DAC.

Previous studies indicated that treatment of adult rat with DAC results in the accumulation of desmosterol in serum and muscle membranes [3]. It was also found that supplemental high cholesterol diet given to rats receiving DAC prevented the appearance of myotonia and the accumulation of desmosterol [3]. Thus, exogenous cholesterol blocks the synthesis of new cholesterol and thereby the accumulation of desmosterol. We could not detect any desmosterol in the DAC-treated muscle cells using gas–liquid chromatography. This could be due to the fact that the growth medium used for muscle cultures in our studies contained cholesterol (1.7 mg/ml) which might have blocked the synthesis of new cholesterol by muscle cells and hence prevented desmosterol accumulation. A similar lack of increase in desmosterol was reported for DAC-treated cultured chick muscle cells [28].

In the present study, a microsomal fraction isolated from DAC-treated cultured muscle cells showed lower levels of adenylate cyclase activity, particularly in response to isoproterenol and NaF. This observation is in accordance with our earlier finding in the plasmalemma of adult rat treated with DAC, where also adenylate cyclase response to isoproterenol and NaF was decreased [18]. However, the β-adrenergic receptors, through which isoproterenol is known to enhance adenylate cyclase, were found to be normal with regard to both density and affinity. Thus, normal levels of β-adrenergic receptor with decreased response of adenylate cyclase to β-adrenergic agonists indicated an altered receptor–enzyme coupling in DAC-treated cells. The defect appeared to be at a step distal to receptor–agonist interaction. We observed that cAMP-phosphodiesterase activity was normal in DAC-treated cells. Hence, a decrease in adenylate cyclase may suggest a lower level of cAMP production in DAC-treated cells. A well recognized defect common to human (both in myotonia congenita and myotonic atrophy), goat, and DAC-induced myotonias appears to be an increase in muscle-fiber plasmalemma resistance with a decrease in its chloride conductance [3, 29]. Cyclic AMP has been shown to enhance chloride transport in certain tissues such as dogfish rectal gland [30] and corneal epithelium [31]. It is not known at present whether cAMP plays a similar role in chloride transport of muscle fiber; if it does, the lowered chloride transport in myotonic muscle could be due to decreased levels of cAMP in those muscle fibers.

The present studies indicated an increase in guanylate cyclase and cGMP-phosphodiesterase in DAC-treated muscle cells, which suggested an increase in the turnover of cGMP. The exact significance of cGMP in skeletal muscle functions is not known at present. In general, cGMP is known to promote cellular differentiation during development, while its counterpart cAMP promotes growth [32]. In many situations, cGMP is known to play a role antagonistic to cAMP [23]. In this respect, it is noteworthy that adenylate cyclase is considerably decreased in our DAC-treated cultured muscle cells. Whether an imbalance between cAMP and cGMP plays a role in inducing myotonic changes in muscle remains unknown.

In conclusion, we have shown that DAC, when applied to muscle cells grown in culture, induces morphological and biochemical changes. However, it is important to note that the present *in vitro* system is not analogous to the DAC-treated myotonic animal model. The studies reported here differ from the animal model in that the present experiments with DAC were carried out with muscle cultures grown in a medium containing exogenous cholesterol, which is known to block the synthesis of new cholesterol and thereby prevents desmosterol accumulation. In the animal model, DAC-induced myotonia is shown to be closely related to the accumulation of desmosterol in serum and muscle membranes [3]; however, myotonia induced by clofibrate does not require desmosterol accumulation [33]. Therefore, further experiments on the effect of DAC and clofibrate on muscle cultures grown in the presence and absence of exogenous cholesterol would be valuable in determining the significance of desmosterol accumulation in this experimental model.

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