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20,25-DIAZACHOLESTEROL, CLOFIBRIC ACID AND 2,4-DICHLOROPHENOXYACETIC ACID---ALL INDUCERS OF MYOTONIA---PERTURB THE POST-LANOSTEROL SECTOR OF STEROL BIOSYNTHESIS IN CULTURED CHICK EMBRYO FIBROBLASTS*

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SUMMARY: When administered to mammals 20,25-diazacholesterol (DAC), clofibric acid (CA) and 2,4-dichlorophenoxyacetic acid (2,4-D) induce myotonia. DAC and CA also interfere with cholesterol metabolism. No such effect has hitherto been observed for 2,4-D and hence there was no support for there being a causal relationship between drug-induced myotonia and impaired cholesterol biosynthesis. Radiogas chromatographic methods are used in this paper to establish that all three agents cause intermediates between lanosterol and cholesterol to accumulate in cultured chick embryo fibroblasts. It is therefore feasible to examine further the possibility that perturbed sterol metabolism is a sufficient and necessary condition for experimentally induced myotonia.

A variety of drugs can induce myotonia in mammals (1). The most well-studied examples are: 20,25-diazacholesterol (DAC), triparanol, zuclomiphene, clofibric acid (CA), 2,4-dichlorophenoxyacetic acid (2,4-D) and indole acetic acid (IAA) (2-10). The first three drugs listed have a pronounced effect on steroid metabolism in whole mammals, causing desmosterol to accumulate in tissue and blood (4,7,11-13). For this reason, it has been proposed (14,15) that myotonia could be a membrane defect resulting from an aberrant sterol content of the sarcolemma. Work with the aromatic carboxylic acids CA, 2,4-D and IAA does not support this proposal unreservedly, however. True CA, or more commonly its easily hydrolysed ethyl ester (16), interferes with sterol metabolism (17) but that interference appears to be focused at the β-hydroxy-β-methylglutaryl coenzyme A reductase level (18). Eberstein, et al. (19) detected no accumulation of desmosterol or other sterols in rats treated with the ethyl ester of CA. To the best of our knowledge, there are no reports extant of either 2,4-D or IAA affecting sterol metabolism.

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In this paper we establish that DAC, CA and 2,4-D, when applied to a common batch of cultured chick embryo fibroblasts in the concentration range 1-10 μg/ml culture medium, decrease cholesterol biosynthesis and lead to an accumulation of acetate-derived isotope in desmosterol and related sterols. This finding lends credence to the proposal that drugs such as those mentioned above produce myotonia by perturbing sterol metabolism.

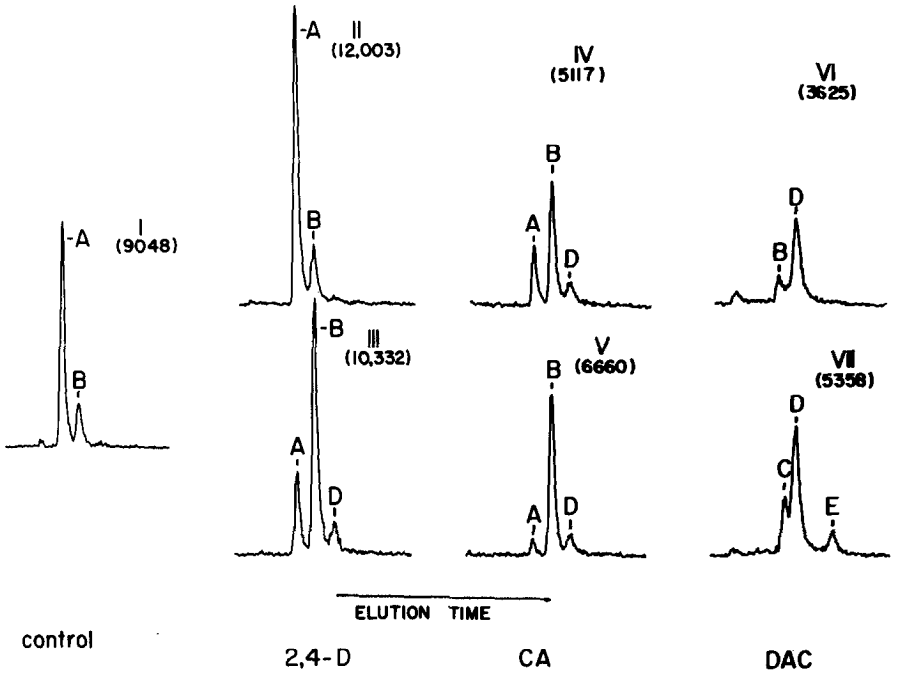
MATERIALS AND METHODS

Embryonic chick fibroblasts were prepared from primary muscle cultures (20) by the following procedure. Primary muscle cultures that were 7 days old were washed twice with buffered saline (Ca^{2+} and Mg^{2+} free) and then incubated at 37° for 10 minutes with a solution of 0.125% trypsin and 3 x 10^{-4} M EDTA (3 ml/15 cm Petri plate). Following addition of 0.25 volumes of horse serum, the detached cells were dispersed by trituration. The cell suspension was filtered through nylon mesh, and was diluted with that volume of the medium of Coleman and Coleman (21) which allowed the cells to be seeded on 15 cm uncoated plastic Petri plates at a density of 5 x 10^6 cells/20 ml. The medium was changed on the 3rd and 5th days. By the 7th day the cells were confluent and were subcultured again. This third generation of cells had medium changed on the 3rd day and were subcultured on the 5th day. The medium of the fourth generation of cells was changed on the 3rd and 5th days and the cells were used on the 6th day (the 25th day since establishing the primary muscle culture).

In all experiments, cells were first depleted of serum for 4.5 hours before being treated with [1-^{14}C] acetate (5 μCi/15 cm plate, 47 μCi/μmole) and the stated level of DAC, 2,4-D or CA in serum-free medium. After 12 hours the cells were washed with buffered saline and the cells collected by scraping/centrifugation. Cells were then homogenized in the biphase ethyl acetate/acetone/water (1:1:1) for 10 minutes. Prior to the homogenization, the internal standard cholestane had been added to the ethyl acetate. The organic layer was removed, was stripped of solvent and the residue was treated briefly with ethereal diazomethane prior to analysis by radiogas chromatography (RGC). The stationary phase in the RGC analysis was 3% SP 2250 on Supelcoport (80-100 mesh) and the analysis was conducted at 300°C. Peaks in the RGC trace were identified by RGC/mass spectrometry (22).

RESULTS

The radioactivity profiles that were obtained from control cells and from cells treated separately with 1 and 10 μg/ml culture medium of DAC, CA and 2,4-D are shown in the Figure. The profiles have been normalized to a constant amount of cellular cholesterol. The instantaneous count rate associated with the highest peak in each profile is noted on each trace in parentheses. Retention index measurements and mass spectra established that radioactivity peaks A, B and C were due exclusively to cholesterol, desmosterol and cholesta-8,24-dien-3-ol respectively. Peaks D and E were composite. In the former there were potential



FIGURE

Profiles of radioactivity obtained by radiogas chromatographic analyses of total organic extracts of fibroblast treated with [1-^{14}C] acetate and the stated dose of one of the drugs for 12 hours. The instantaneous count rate associated with the largest peak in each profile is noted in parentheses. Peaks A, B and C are caused respectively by cholesterol, desmosterol and cholesta-8,24-dien-3-ol; peaks D and E are composite (see text). Panel code: I, control; II, 1 μg 2,4-D/ml (4.5 x 10^{-6}M); III, 10 μg 2,4-D/ml (4.5 x 10^{-5}M); IV, 1 μg CA/ml (4.7 x 10^{-6}M); V, 10 μg CA/ml (4.7 x 10^{-5}M); VI, 1 μg DAC/ml (2.6 x 10^{-6}M) and VII, 10 μg DAC/ml (2.6 x 10^{-5}M).

contributions from cholesta-7,24-dien-3-ol, cholesta-5,7,24-trien-3-ol and a 4-methylcholesta-8,24-dien-3-ol; in the latter there were contributions from lanosterol, 4,4-dimethylcholesta-8,24-dien-3-ol and a 4,4-dimethy-cholestatrienol.

The Figure demonstrates that in control cells (panel I) acetate-derived isotope was transferred exclusively to cholesterol (79.6% of total radioactivity incorporated into free sterols) and desmosterol (20.4% of total sterol activity). Although the data are not shown in the Figure, the mass of the desmosterol pool in the cells was 0.022 times that of cholesterol. A dose of 1 μg 2,4-D/ml had no noticeable effect (panel II), cholesterol contained 78.9% and desmosterol 21.1% of total sterol activity and the desmosterol/cholesterol mass ratio was 0.023.

Increasing the 2,4-D dose to 10 μg/ml (panel III) had a marked effect on sterol metabolism in fibroblasts. The bulk of the acetate-derived isotope was transferred to desmosterol (67.8%) rather than cholesterol (21.2%) and the desmosterol/cholesterol ratio increased to 0.081. A third peak of radioactivity (peak D) which was of composite origin (see above) now also contained a significant amount (11.1%) of the incorporated radioactivity. Clearly, 2,4-D can perturb sterol metabolism in the post-lanosterol sector of the biosynthetic pathway.

CA behaves similarly but with higher efficiency. A dose of 1 μg/ml (panel IV) led to there being 25.0% of the incorporated activity in cholesterol, 60.3% in desmosterol and 14.8% in peak D (desmosterol/cholesterol ratio, 0.059). A dose of 10 μg/ml (panel V) accentuates this distribution: cholesterol (6.1%), desmosterol (79.5%) and peak D (14.4%), desmosterol/cholesterol ratio (0.065) .

Panels VI and VII contain the DAC results. As had been seen previously in cultured chick muscle cells (20), doses of 1 and 10 μg DAC/ml inhibited completely the biosynthesis of cholesterol and led to accumulations of isotope in peak D. The 1.0 μg DAC/ml dose led to 17.1% of incorporated activity being in desmosterol and 82.9% in peak D (desmosterol/cholesterol ratio; 0.030). In the 10 μg DAC/ml dose, the desmosterol percentage dropped to zero, peak D now contained 68.2% of total incorporated radioactivity with the balance being found in cholesta-8,24-dien-3-ol (peak C, 20.2%) and the composite peak E (11.7%). The desmosterol/cholesterol ratio in the 10 ug DAC/ml run was 0.026.

DISCUSSION

The results demonstrate clearly that 2,4-D, CA and DAC when administered to cultured chick embryo fibroblasts at a level of 10 μg/ml culture medium disrupt the flow of acetate-derived isotope in the post-lanosterol sector of the cholesterol biosynthetic pathway. In the case of 2,4-D and CA, this disruption leads predominantly to an accumulation of isotope and mass in desmosterol; with DAC, intermediates less proximal to cholesterol accumulate. For CA and DAC, but not for 2,4-D, a dose of 1 μg/ml also have this effect. These results are

consistent with the hypothesis that myotonia induced by azasteroids, steroid "analogs" such as triparanol and some aromatic monocarboxylic acids results from an altered steroid composition of the sarcolemma resulting from a perturbed sterol composition of the latter.

Further work has to be done to define rigorously which sterol biosynthesis enzymes are inhibited by the drugs tested. The results of the isotope incorporation experiments here reported, suggest that both 2,4-D and CA affect the Δ^{24}-reductase while DAC affects the enzymes responsible for the rearrangement of the Δ^{8}-double bond in ring B to the Δ^{5} -position. DAC has a similar effect in cultured chick muscle cells (20).

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