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Original Article

Cytochalasin-D treatment triggers premature apoptosis of insect ovarian follicle and nurse cells

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ABSTRACT Follicle and nurse cells of developing lepidopteran ovarian follicles are eliminated after oocyte maturation. The process of disintegration of both cell types can be triggered prematurely in the follicle development by *in vivo* or *in vitro* treatment with a selective anti-actin agent cytochalasin D. Morphological changes observed in both follicle and nurse cells after cytochalasin D administration at the light and electron microscopy levels reveal all the characteristic morphological markers of a process called apoptosis, or programmed cell death. These changes include detachment of affected cells from basal lamina, loss of microvilli, crowding of structurally intact organelles, condensation of cytoplasm, nuclear shrinkage and fragmentation, and chromatin condensation. Examination of genomic DNA isolated from cytochalasin D affected cells revealed internucleosomal DNA fragmentation — a major biochemical hallmark of apoptosis. Experiments involving administration of actinomycin D or cycloheximide, respectively, indicate that the cell death of follicle and nurse cells triggered by cytochalasin D action does not require new RNA and/or protein synthesis. Possible mechanisms by which cytochalasin D could initiate the lethal biochemical pathway of programmed cell death in both cell types are discussed.

KEY WORDS: *programmed cell death, DNA fragmentation, cytochalasin D, actin cytoskeleton, Manduca sexta*

Introduction

Developing follicles in the meroistic polytrophic ovaries of tobacco hawkmoth *Manduca sexta* consist of three different, highly specialized cell types. Two of them, the nurse cells and the oocyte, have their origin in the germ line. A primordial cell, or cystoblast, undergoes three mitotic divisions which result in eight morphologically and genetically indistinguishable cells (Rasmussen and Holm, 1982; King and Büning, 1985). All eight cells, called cystocytes, are interconnected via cytoplasmic bridges forming a syncytium. As oogenesis progresses, one cell becomes an oocyte and the remaining seven cystocytes differentiate into nurse cells (trophocytes), capping the anterior end of the follicle.

The nurse cells and oocyte itself are surrounded by a monolayer of epithelial follicle cells. Unlike nurse cells and oocyte, the follicle cells have their origin in mesoderm. All three types of cells in developing lepidopteran follicle — nurse cells, oocyte and follicle cells — are extremely specialized with respect to their functions during oogenesis. Probably the major role of nurse cells is to synthesize maternal RNA and facilitate its transport to the developing oocyte (Capco and Jeffery, 1979; Watson *et al.*, 1993). The DNA content of the nurse cell nuclei is highly overreplicated reaching a ploidy level of 2^{16} in some lepidopteran species (Berry, 1982; Cardoen *at al.*, 1990). This extreme amount of nuclear genomic DNA in giant amoeboid nuclei provides the nurse cells with the machinery for the synthesis and transport of large quantities of maternal RNA into the oocyte.

The oocyte itself is transcriptionally inactive until the stage of midblastula transition after fertilization, and also no translation can be detected in the developing egg (Kastern *et al.*, 1982). Therefore, all materials necessary for oocyte development must be synthesized in cells other than the oocyte. These sites of synthesis include nurse cells (maternal RNA), follicle cells (chorion proteins and some vitellogenins), and fat body (vitellogenins and other proteins), and these products are transported to the oocyte by various mechanisms (Berry, 1985; Kunkel and Nordin, 1985).

The highly specialized follicle epithelial cells undergo a series of complex changes during their developmental program. Follicle cells help to facilitate the uptake of various materials from hemolymph to the oocyte, and are involved in the synthesis and transport of some oocyte components. Perhaps the major function of follicle cells is to synthesize and deposit the structurally and biochemically

Abbreviations used in this paper: CD, cytochalasin D; DMSO, dimethylsulfoxide; DNase I, deoxyribonuclease I; EDTA, ethylenediaminetetraacetic acid; F-actin, filamentous actin; G-actin, monomeric actin; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TX, Triton X-100.

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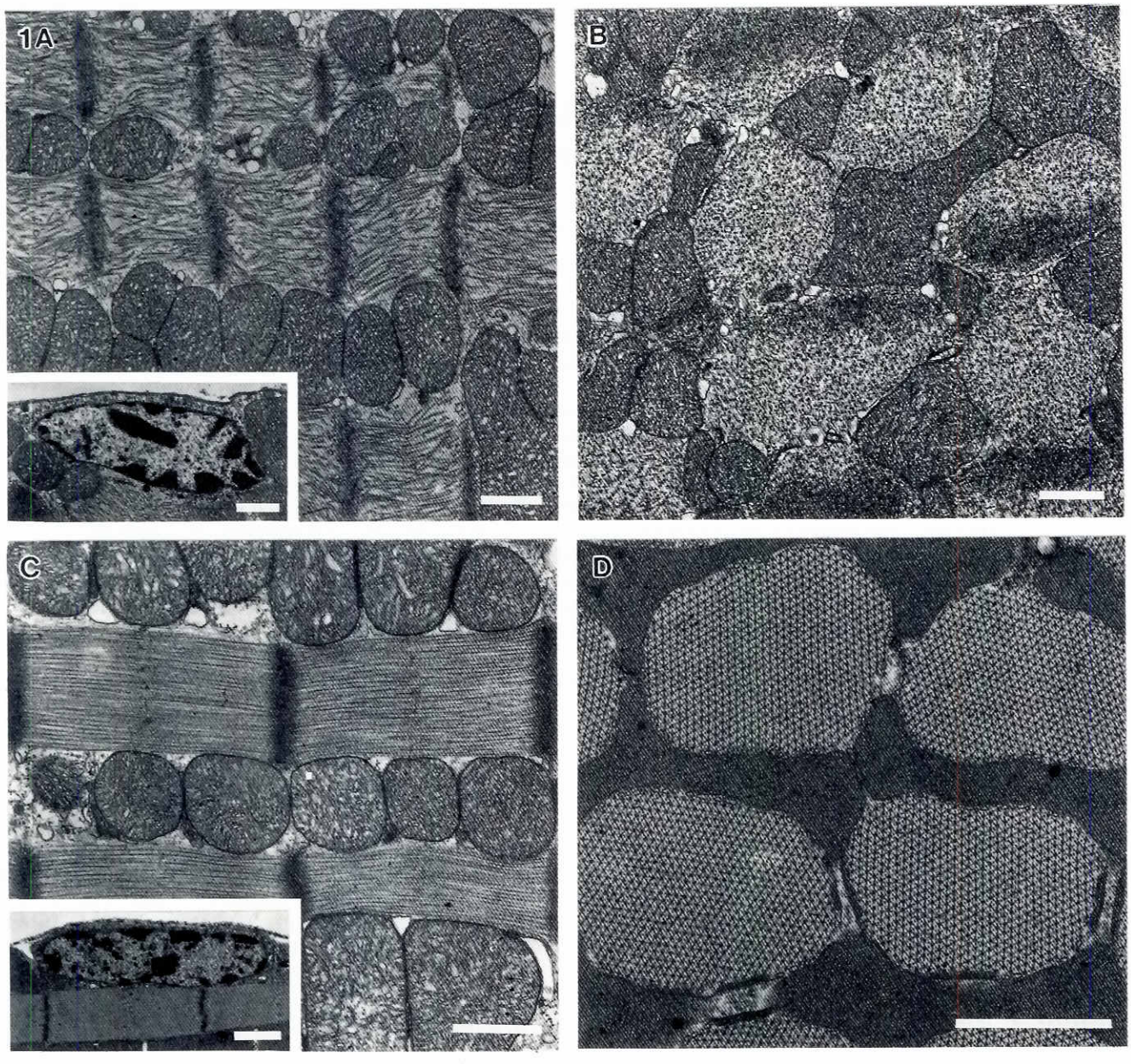

Fig. 1. Effect of CD on indirect flight muscles of males and females of the hawkmoth *M. sexta*. **(A)** *Longitudinal section of muscle tissue of a hawkmoth male treated in vivo with CD for 4 h shows disintegration of sarcomeric filaments. Mitochondria, sarcoplasmic reticulum, as well as nuclei (inset of Fig. 1A) appear to be intact.* **(B)** *The cross-section of the same preparation illustrates randomized organization of muscle filaments after CD treatment.* **(C)** *The indirect flight muscle tissue of M. sexta female injected with CD does not reveal any detectable morphological changes in its ultrastructure. The sarcomeres in myofibrils, as well as mitochondria, sarcoplasmic reticulum and nuclei (inset of Fig. 1C) have ultrastructure indistinguishable from control untreated individuals (data not shown).* **(D)** *The cross-section through "AI" region of a female sarcomere shows regular hexagonal arrangement of the muscle filaments even 4 h after in vivo CD treatment. The scale bars represent 1 μm*

very complex eggshell, or chorion, after the completion of the vitellogenic phase of oogenesis (for review, see Kafatos *et al.*, 1977; Margaritis, 1985). The first eggshell product of the follicle cells is a vitelline membrane, which is secreted immediately after the degeneration of the nurse cells. Subsequently, three chorion layers are formed, surrounding entire egg surface. The DNA in the follicle cell nuclei is also highly overreplicated, although the ploidy level is not as extreme as in the nurse cells and the nuclei are rather spherical than amoeboid in shape. The enhanced DNA content in the follicle cell nuclei is probably necessary to facilitate the synthesis of considerable amounts of chorion proteins.

The final step of both the nurse cell and follicle cell developmental program is their programmed disintegration after they complete their functions in oogenesis. At present there is little information available about the exact mechanism by which these cells are eliminated after oocyte maturation. It is very likely, however, that

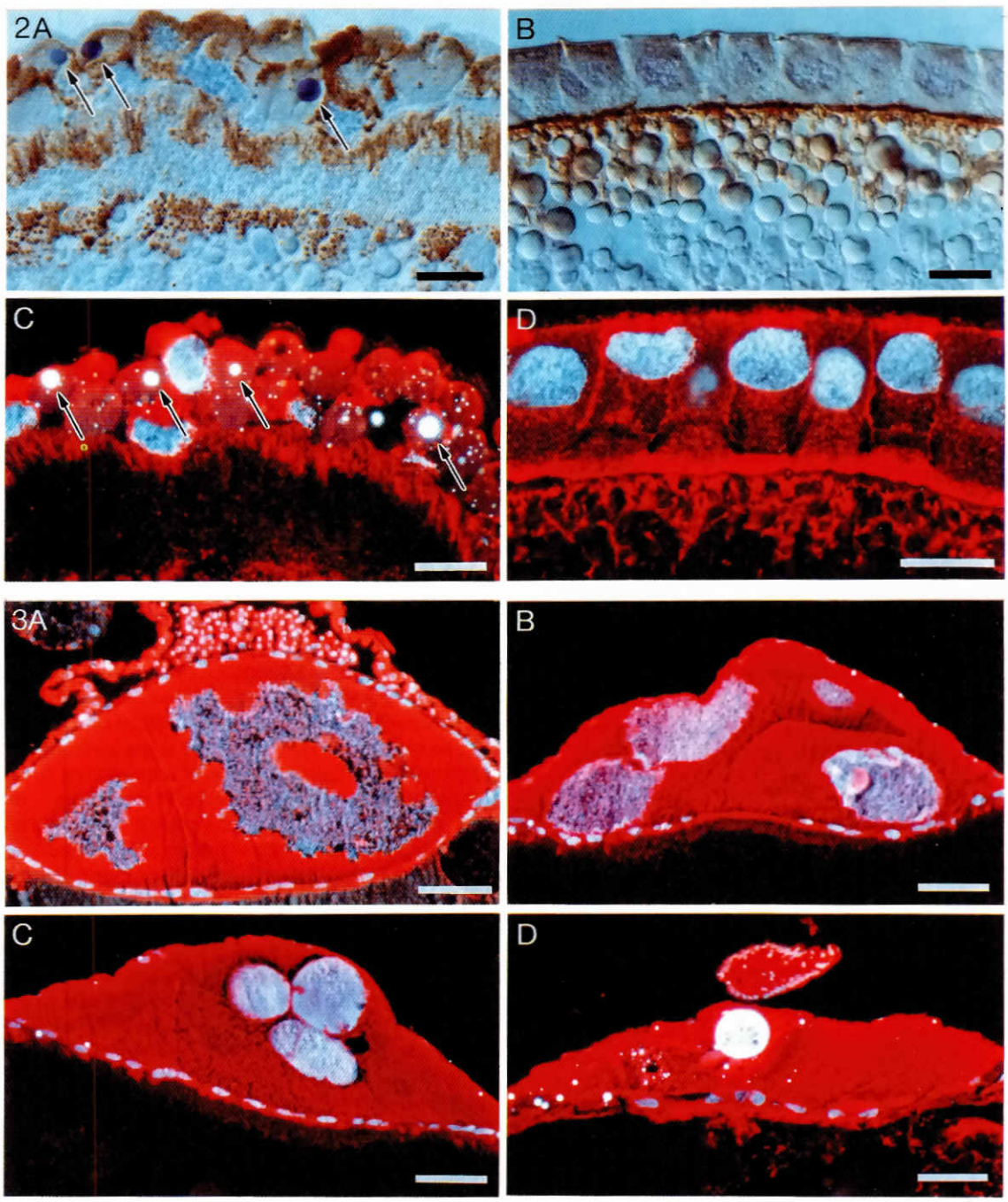

Fig. 2. Follicle cells and the cortical region of a developing oocyte of *M. sexta*. **(A)** *Nomarski image of a section through developing ovarian follicle treated in vitro with CD for 6 h. Some nuclei of the follicle cells are shrunk and very intensively stained with hematoxylin (arrows). The brown color represents disrupted actin-based cortical cytoskeleton visualized using monoclonal antibody.* **(B)** *Control section of an untreated follicle stained as described above.* **(C)** *In vivo CD treated developing follicle stained specifically for DNA using Hoechst 33342 fluorescent dye. Collapsed nuclei of some follicle cells (arrows) show very strong signal in comparison to the normal nuclei (Fig. 2D). Actin-based cytoskeleton was visualized immunohistochemically with monoclonal antibody coupled to Texas red fluorescent dye.* **(D)** *Control untreated ovarian follicle stained as described above. The scale bars represent 20 μm.*

Fig. 3. Progressive morphological changes and chromatin condensation of giant nurse cell nuclei after *in vivo* CD treatment. *DNA was detected using specific fluorescent dye Hoechst 33342, the red color represents cytoplasmic actin visualized with monoclonal antibody and Texas red fluorophore.* **(A)** *Control section through the nurse cell cap of an untreated developing follicle of M. sexta.* **(B,C,D)** *Nurse cell caps of follicles treated in vivo with CD for 2, 4, and 8 h, respectively. Increasing brightness of DNA staining in these preparations demonstrates progressive chromatin condensation after CD treatment. The scale bars represent 50 μm.*

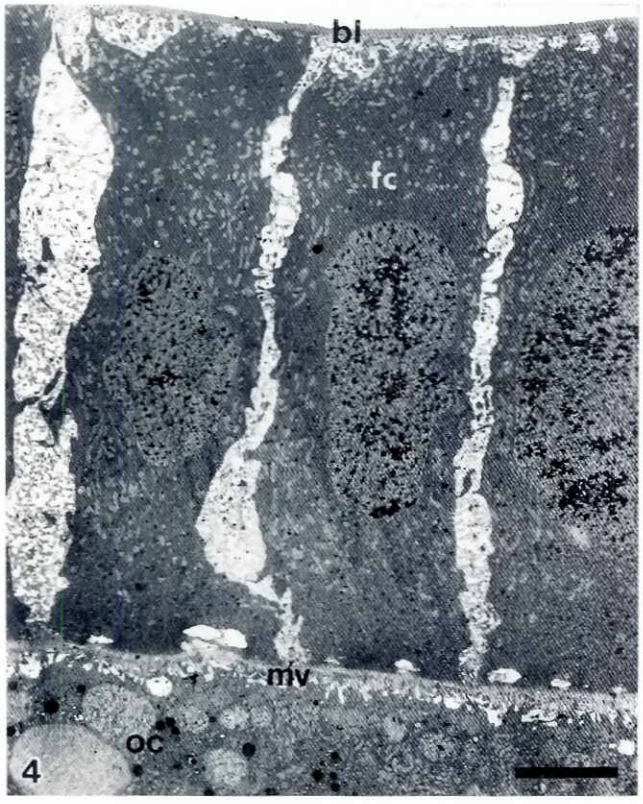

Fig. 4. Electron micrograph of normal untreated follicle epithelial cells of *M. sexta* developing ovarian follicle. *bl, basal lamina; fc, follicle cell; mv, microvilli; oc, oocyte. The scale bar represents 2 μm.*

they are terminated by a process called apoptosis — programmed cell death. In this manuscript we report that both *in vivo* and *in vitro* treatment of *Manduca* developing follicles with a selective anti-actin agent, cytochalasin D (CD), appears to trigger apoptosis in both the nurse cells and the follicle cells much earlier in the ovarian follicle development, even before they complete their physiological functions. Moreover, these specific degenerative effects of CD are confined to these two cell types in the developing follicle.

Results

Effect of cytochalasin D on muscle cells

Both vitellogenic females and mature males of the hawkmoth *Manduca sexta* were injected in the abdomen with CD, an agent which has been shown to have a selective inhibitory and disruptive effect on actin based structures (Selden *et al.*, 1980; Schliwa, 1982). Injected females survived normally for several days and were able to copulate and lay eggs. Mature males injected with the same dose of CD, however, died usually within several hours. The tissue which would seem to be a primary target of CD action would normally be muscle because of its high actin content.

Fig. 1A represents a longitudinal section of indirect flight muscle tissue of CD injected male of *Manduca sexta*. This transmission electron micrograph shows the disintegration of muscle filaments in the sarcomeric regions of myofibrils. In contrast, the sarcoplasmic reticulum, the mitochondria and the nuclei (inset of Fig. 1A) appear to be unaffected by the CD treatment. Fig. 1B demonstrates the randomized organization of muscle filaments in a cross section through the "AI" zone of a sarcomere after CD injection.

Interestingly, the *in vivo* CD treatment of *M. sexta* females revealed no detectable effect on indirect flight muscle tissue structure at both light microscopy and electron microscopy levels. Both longitudinal and cross sections examined by transmission electron microscopy showed the normal distribution of muscle filaments in sarcomeres, the same pattern as demonstrated in the control tissue without CD treatment (data not shown), as well as intact structures of muscle cell nuclei and other organelles. The ultrastructure of indirect flight muscle tissue from a female injected with CD is shown in Fig. 1C,D. The longitudinal section (Fig. 1C) illustrates the normal organization of sarcomeres in the muscle tissue of CD treated female; the inset of Fig. 1C shows an intact nucleus of a muscle cell. The cross section (Fig. 1D) demonstrates the hexagonal arrangement of actin and myosin filaments at the level of the "AI" zone of a sarcomere.

Collapse of nurse and follicle cell nuclei after cytochalasin D treatment

The effects of *in vivo* CD treatment on the female ovary seem to be very specific, and are confined to the follicles which are undergoing rapid vitellogenesis at the time of injection. Already chorionated mature eggs as well as previtellogenic follicles appear unaffected. If a female injected with CD is allowed to copulate, she will lay normal eggs followed by empty follicles and finally again normal eggs. These three groups of eggs represent respectively, follicles which were fully chorionated before CD injection, follicles in the stages of vitellogenesis, and previtellogenic follicles.

The initial observation of the effect of CD on the female ovarian nurse and follicle cells was made at the light microscopy level. Fig. 2A shows a paraplast section of a developing follicle of *M. sexta* treated *in vitro* with CD. After 4 h of incubation in the presence of CD, most nuclei of the follicle cells are shrunk into one or more compact spheres with the diameter approximately one third of their original size. The intensive staining of these collapsed nuclei with hematoxylin, in comparison to the regular size nuclei, suggests the presence of condensed chromatin. The shape of entire follicle cells changed after CD treatment from the original columnar into more or less spherical structures. Actin immunostaining using monoclonal antibody demonstrates the CD induced disruption of the actin based cortical cytoskeleton in the ovarian follicle (Fig. 2A). The control section of the developing ovarian follicle (Fig. 2B) shows the normal columnar shape of follicle cells, the large spherical follicle cell nuclei, as well as an intact cortical cytoskeleton visualized using monoclonal antibody against cytoskeletal actin.

The presence of DNA in both follicle and nurse cell nuclear remnants after CD treatment has been confirmed using DNA-specific fluorescent stain Hoechst 33342. Fig. 2C represents a fluorescent micrograph of a paraplast section of a developing follicle of *M. Sexta* treated *in vivo* with CD. The section was stained for the presence of DNA using Hoechst 33342 fluorescent dye. As previously demonstrated with hematoxylin staining (Fig. 2A), the shrunk nuclei of follicle cells show a very strong signal, much more intense than normal nuclei (Fig. 2D), confirming the presence of highly condensed chromatin. The immunofluorescent staining of cytoskeletal actin demonstrates the disruption of actin cortical cytoskeleton in the CD treated follicles (Fig. 2C), as opposed to the control preparation in Fig. 2D. Fig. 3A-D illustrate the progress of nuclear shrinkage and chromatin condensation of the giant nurse

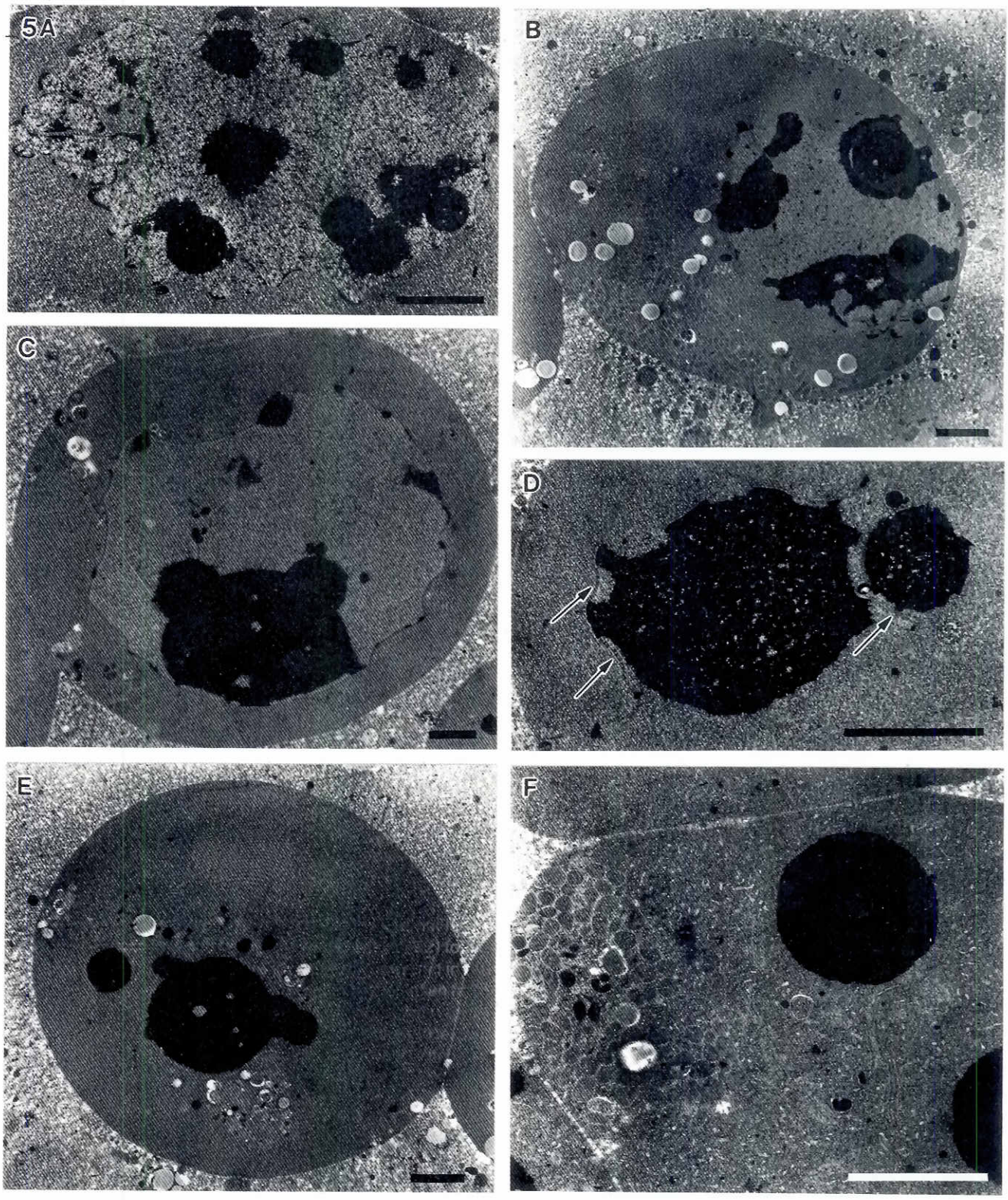

Fig. 5. Progressive changes of the follicle cell ultrastructure after *in vivo* CD treatment. (A) *Chromatin condensation and nuclear envelope involutions are evident 2 h after CD injection.* **(B,C)** *Follicle cells become more or less spherical with clusters of intact organelles close to the cell surface, and the condensation of chromatin progresses 4 and 6 h after CD treatment, respectively.* **(D)** *By 8 h after CD injection, condensed chromatin forms very dense masses which are still partially surrounded by double nuclear membrane (arrows).* **(E,F)** *In the final stage (12-16 h after CD injection), condensed nuclear chromatin breaks into several discrete, usually spherical and very dense structures. Clusters of morphologically intact organelles are still detectable in the cytoplasm at this stage. The scale bars represent 3 μm.*

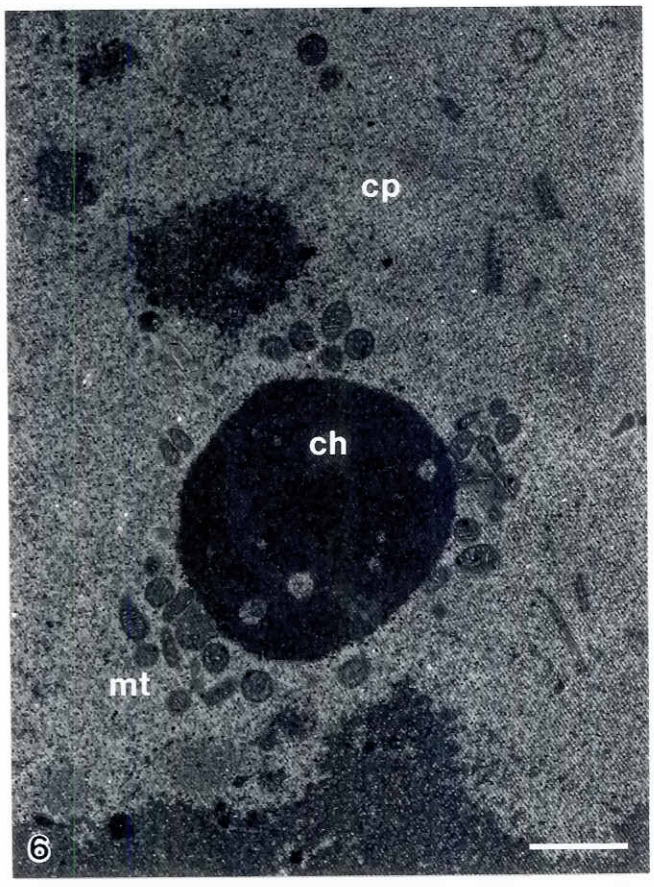

Fig. 6. Ultrastructure of an untreated nurse cell undergoing natural disintegration after oocyte maturation. *The characteristic features include nuclear fragmentation and chromatin condensation (ch), crowding of structurally intact organelles (mt), and condensation of cytoplasm (cp). The scale bar represents 2 μm.*

cell nuclei after *in vivo* CD treatment. The original amoeboid shape of the nurse cell nuclei (Fig. 3A) changes into more or less spherical structures with progressive chromatin condensation as demonstrated by increase of the intensity of Hoechst 33342 DNA staining in these nuclei (Fig. 3B-D). In contrast to the oocyte cortical cytoskeleton, the distribution of cytoskeletal actin in the nurse cell cytoplasm does not seem to be affected by the CD treatment, presumably because most of the actin in the nurse cell is present in a monomeric form (G-actin; Watson *et al.*, 1993). The same results as described above were obtained in experiments, where the *in vivo* or *in vitro* administration of CD was supplemented with actinomycin D or cycloheximide, respectively (not shown).

The ultrastructure of collapsed nurse and follicle cell nuclei

Fig. 4 represents the ultrastructure of intact ovarian follicle cells of *M. sexta* with their characteristic columnar shape, numerous microvilli at the follicle cell-oocyte border, attachment of follicle cells to the basal lamina, cytoplasmic connection between adjacent follicle cells, and large, elliptical nuclei.

The progressive morphological changes of the follicle cell ultrastructure after *in vivo* CD treatment are illustrated in Fig. 5. The follicle cells lose their columnar shape as well as microvilli at the oocyte border, detach from the basal lamina and become more or less spherical structures. The cytoplasm of follicle cells appears to be condensed and crowded with organelles, which retain their integrity, and are usually located close to the cell surface. Two hours after CD injection (Fig. 5A), the most characteristic feature of the CD affected cells is aggregation of nuclear chromatin in relatively large granular masses, often abutting on the nuclear membrane. The nuclear membrane itself is abnormally convoluted at this point, and in some places grossly indented (Fig. 5A,B). Compact, structurally very fine and usually spherical masses of uncertain origin, with slightly less dense material than condensed chromatin, are present in the nuclei throughout all the stages of nuclear disintegration. In the later stage of CD treated follicle cells (Fig. 5C), the masses of condensed chromatin become even larger and also the convolutions of the nuclear membrane are more dramatic (Fig. 5D). Clusters of intact cellular organelles are still present in the condensed cytoplasm. In the final stage (Fig. 5E,F), the nuclei of the follicle cells have broken up into several discrete, usually spherical fragments, some of which are at least partly surrounded by double membrane. Even in this stage, the cellular organelles are still clearly visible in the highly condensed cytoplasm, and most of them appear to be structurally intact. The same morphological changes at electron microscopy level in both follicle cells and nurse cells were obtained when CD treatment was supplemented with actinomycin D or cycloheximide, respectively (not shown). Fig. 6 represents characteristic ultrastructure of a nurse cell from the mature ovarian follicle undergoing natural disintegration. The structurally intact mitochondria, chromatin condensation, nuclear fragmentation and condensation of cytoplasm clearly suggest that these cells are eliminated from mature oocyte by an apoptotic process very similar to the CD induced one, after they complete their physiological functions in oogenesis.

Internucleosomal DNA fragmentation of cytochalasin D treated follicle and nurse cells

The effect of CD treatment on the follicle and nurse cell genomic DNA has been examined by means of agarose gel electrophoresis. The results of four electrophoretically separated DNA preparations are shown in Fig. 7. Lane 1 represents the genomic DNA isolated from control, untreated developing ovarian follicles of *M. sexta*. One intensive band with molecular weight in the range over 50 kilobases is clearly visible, and no lower molecular weight fragments of DNA can be detected in this control preparation. The DNA isolated from developing follicles 4 h after CD injection and separated on agarose gel is shown in lane 2. The DNA step ladder, a major biochemical marker of the process of programmed cell death (Compton, 1992), clearly demonstrates the internucleosomal DNA fragmentation after *in vivo* CD treatment. Lanes 3 and 4 in Fig. 7 show the progressive degradation of high molecular weight DNA isolated from *M. sexta* ovarian follicles 8 and 16 h after CD injection, respectively.

Discussion

A fungal metabolite, cytochalasin D, has been shown to have a variety of actions on living cells (Miranda *et al.*, 1974; Mookerjee *et al.*, 1981; Schliwa, 1982). Both *in vivo* and *in vitro* studies have demonstrated that the primary, and probably the only target of CD action is actin. Cytochalasins A and B, by contrast, also interfere with monosaccharide transport mechanism across the plasma membrane by competing for hexose membrane receptor (Yahara *et*

al., 1982; Goddette and Frieden, 1986a; Cooper, 1987). Cytochalasin D, unlike most of other cytochalasins, can not only inhibit the actin polymerization by "capping" the barbed (faster-growing) end of microfilaments, but also has a strong disruptive effect on the existing actin filaments (Selden *et al.*, 1980; Mozo-Villarias and Ware, 1984; Walling *et al.*, 1988). The inhibitory effect of CD on actin polymerization is about an order of magnitude higher than that of cytochalasin B, the most frequently used cytochalasin in actin studies (Brown and Spudich, 1979; Cooper, 1987). In addition, CD is also able to bind to monomeric actin (G-actin) and to accelerate the initial rate of filamentous actin (F-actin) assembly, indicating more complex interactions between actin and CD than just simple inhibitory and disruptive effect (Goddette and Frieden, 1986b; Walling *et al.*, 1988; Wilder and Ashman, 1991).

In our experiments, we describe the disruptive effect of CD on *M. sexta* male indirect flight muscle tissue. The disintegration of sarcomere organization as opposed to the intact structure of other muscle cell organelles, including nuclei, demonstrates the specificity of CD action on F-actin. A surprising phenomenon, however, is the fact that in contrast to males, no detectable effect of *in vivo* CD treatment was observed on the female muscle tissue. In addition, the females of *M. sexta* survived the CD administration for long periods of time in contrast to males, which died within several hours after CD injection. Vitellogenic females of lepidopterans synthesize most, if not all, of their vitellogenins and some other proteins necessary for oocyte development in fat body. These components are then transported through hemolymph and taken up by oocytes by a process of endocytosis (Telfer, 1960; Kunkel and Pan, 1976; Kunkel and Nordin, 1985). This oocyte uptake mechanism very likely represents the basis for the resistance of hawkmoth females to CD treatment, as opposed to males. However, differences in malpighian tubule excretion or fat body detoxification cannot be ruled out. It has been previously reported that in addition to yolk proteins, a considerable amount of actin is synthesized by fat body of moth vitellogenic females, and its transport and acquisition by oocytes is similar to that of vitellogenins (Jarnot *et al.*, 1988). Since the CD can bind also G-actin, the uptake of actin from hemolymph together with bound CD can represent a detoxifying mechanism of *M. sexta* females and explain the lack of detectable effects of CD on their muscle tissue. An alternative possibility is a nonspecific acquisition of CD by the oocytes, since almost any component present in the hemolymph of vitellogenic females has been demonstrated to be taken up by developing follicles (Jarnot *et al.*, 1988). Since CD is a small molecule which can permeate plasma membranes (Cooper, 1987), this process of CD elimination from the female hemolymph must be relatively very fast and efficient, because prolonged exposure to CD in the hemolymph would damage surrounding tissue, including indirect flight muscles, as observed in CD injected males.

Histological examination of developing vitellogenic follicles of *M. sexta* treated *in vivo* or *in vitro* with CD revealed dramatic changes in the structure of both follicle and nurse cells. At the light microscopy level, the most characteristic features of CD treatment are loss of the original columnar shape of follicle cells and their conversion to rather spherical structures, and considerable shrinking of both follicle and nurse cell nuclei. Transmission electron microscopy observations illustrate the ultrastructure of progressive changes of the CD affected cells of developing follicles, including chromatin condensation, nuclear fragmentation, detachment of cells from basal lamina, disappearance of microvilli, condensation

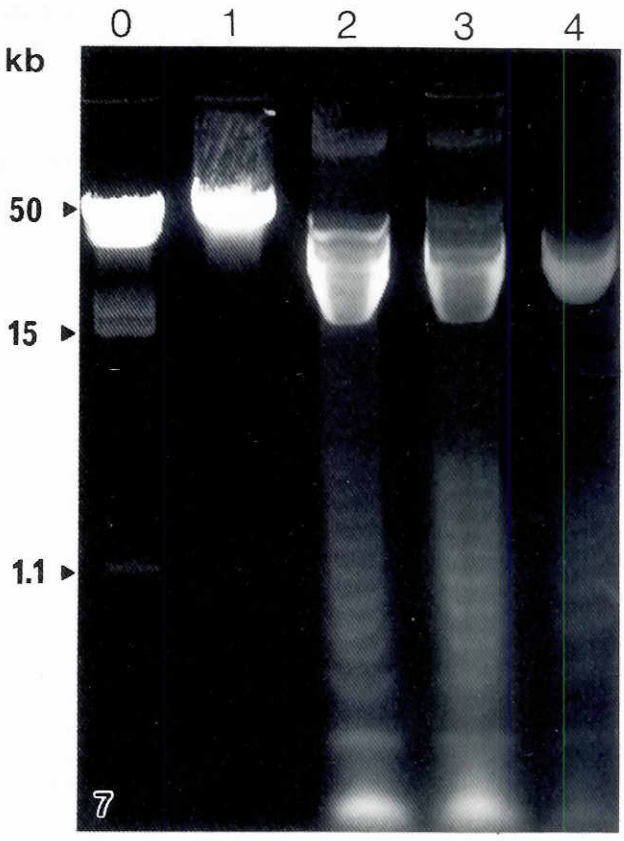

Fig. 7. Agarose gel electrophoresis of genomic DNA isolated from developing follicles of *M. sexta*. *Lane 1 represents control genomic DNA from untreated ovarian follicles. Lanes 2, 3, and 4 demonstrate internucleosomal DNA fragmentation producing characteristic step ladder pattern 4, 8, and 16 h after in vivo CD treatment, respectively. The molecular weight marker (I DNA mixed digest) was loaded in line 0.*

of cytoplasm, and crowding of structurally intact organelles. All these morphological events in CD-treated follicles revealed by light and electron microscopy are known to be cardinal characteristic features of cells which are dying by a process called apoptosis or programmed cell death (for review, see Wyllie *et al.*, 1980; Gerschenson and Rotello, 1992). The internucleosomal genomic DNA fragmentation demonstrated by agarose gel electrophoresis of DNA isolated from CD affected cells represents the most characteristic biochemical hallmark of the process of apoptosis (Compton, 1992). This result supports the histological observations and clearly indicates that both *in vivo* and *in vitro* CD treatment triggers the process of programmed cell death in the cells of the developing follicle of hawkmoth *Manduca sexta*, which would normally be eliminated much later in the oogenesis.

A number of studies have examined the process of apoptosis in insects, mostly in *Drosophila* and several Lepidopteran species (Smith and Nijhout, 1982; Fischbach and Technau, 1984; Truman, 1984; Campos-Ortega and Hartenstein, 1985; Lockshin, 1985; Bryant, 1988; Kimura and Truman, 1990; Clem *et al.*, 1991; Wolff and Ready, 1991; Campos *et al.*, 1992, Abrams *et al.*, 1993). Although these studies described programmed cell death in different cell types, for example neurons, intersegmental muscle cells and prothoracic glands, the characteristic morphological markers were

very similar in all examined cell types and are consistent with most of the ultrastructural features we have found in *Manduca* apoptotic follicle and nurse cells.

Programmed cell death, often called apoptosis (Alles *et al.*, 1991), is a very complex, genetically controlled, energy-dependent process in which specific cells are eliminated without compromising the integrity of the rest of the organism (Kerr *et al.*, 1972). Unlike necrotic cytolysis, cells dying by apoptosis do not induce an inflammatory reaction, even if they are present in relatively large numbers. Apoptosis has been shown to be very common during normal embryonic development and metamorphosis of both vertebrates and invertebrates (Glücksmann, 1951; Saunders, 1966, or for review, see Gerschenson and Rotello, 1992). Programmed cell death has been proposed to be an effective way to selectively eliminate single or even large numbers of cells after completion of their functions without any damage to surrounding tissue (for reviews, see Kerr *et al.*, 1987; Wyllie, 1987; Waring *et al.*, 1991).

The process of apoptosis in normal development is known to be modulated by exogenous stimuli, for example steroid and peptide hormones (Fesus *et al.*, 1991; Compton and Cidlowski, 1992). Two alternative models have been proposed for molecular regulation of program cell death. In the first one, the exogenous stimuli activate transcription of a specific gene (or set of genes) and/or *de novo* synthesis of intracellular proteins which are lethal to the same cell. The second model does not require *de novo* protein synthesis, but it involves existence of an intracellular repressor of apoptosis whose inactivation initiates the biochemical pathway of the cell death. An alternative of this model is the presence of an intracellular inducer of apoptosis which is then activated by appropriate stimuli (for review, see Owens and Cohen, 1992). Both models have been supported by experimental evidence, and the two types of apoptosis regulation may be utilized in different cell types or by the same cell under different physiological conditions (Cohen and Duke, 1984; Martin *et al.*, 1990).

The mechanism by which CD triggers the apoptosis of follicle and nurse cells is not clear. It is very likely that both cell types possess a genetic program which would cause them to be eliminated by the process of apoptosis later in the history of the cells, rather than by a costly and potentially hazardous necrosis, after the completion of their functions in oogenesis. We suggest that this process can be prematurely activated by the action of CD, and that it may not require new transcription and/or *de novo* protein synthesis, because neither actinomycin D, nor cycloheximide prevents the CD induced apoptosis of follicle and nurse cells. Therefore, our experimental results support the second model mechanism of regulation of programmed cell death. Since the primary target of CD action is actin, one possible mechanism for triggering the apoptosis could involve a disruption or altering of an actin based cytoskeleton. It has been well established that microfilaments play a crucial role in transport, sorting, and positioning of mRNA and some critical proteins, including enzymes, in the cell cytoplasm (Sundell and Singer, 1991; Sauman *et al.*, 1992; Taneja *et al.*, 1992; Watson *et al.*, 1993). The modifying effect of CD on follicle and nurse cell actin cytoskeleton could induce a lethal biochemical pathway, whose necessary components are already present in the target cells. Some of these components might interact either directly or indirectly with actin based cytoskeleton. This hypothesis would explain the fact that CD triggers apoptosis in susceptible nurse and follicle cells, but fails in the case of male indirect flight muscle cells, which obviously lack the necessary components for biochemical pathway of programmed cell death in their cytoplasm. Another feasible explanation for CD action is based on a recent discovery which identified the endonuclease involved in the internucleosomal cleavage of apoptotic cell genome as deoxyribonuclease I (DNase I; Peitsch *et al.*, 1993). It has been well established that DNase I endonuclease activity is completely inhibited by equimolar binding of the enzyme to G-actin (Lazarides and Lindberg, 1974; Hitchcock, 1980). In agreement with the known CD binding affinity to G-actin, the CD might compete with actin inactivated DNase I present in the cytoplasm or nuclei for G-actin, and, consequently, initiate genomic DNA fragmentation by DNase I activation.

Although it has been reported that disruption of actin filaments may interfere with formation of apoptotic bodies in the final stage of programmed cell death (Cotter *et al.*, 1992), this paper presents first evidence for involvement of F-actin in the process of initiation and possibly regulation of apoptosis. Since the only demonstration of internucleosomal DNA fragmentation in any invertebrate was obtained with baculovirus infected Lepidopteran cell lines (Clem *et al.*, 1991), this work presents the first evidence that it can occur in a tissue as well, albeit a pharmacologically treated one. Further research will be necessary to better understand not only a very complicated machinery of regulation of programmed cell death, but also the molecular mechanisms of CD actions on highly specialized and physiologically very active cells in a complex multicellular organism.

Material and Methods

Animals

Adult males and females of the hawkmoth, *Manduca sexta*, were obtained from our laboratory colony. The animals were reared under standard 16L:8D light-dark conditions at 27±1°C. Larvae were raised in individual containers and fed an artificial diet as described elsewhere (Bell and Joachim, 1976).

Cytochalasin administration

Cytochalasin D (CD; Sigma, St. Louis, MO, USA) was originally dissolved in dimethylsulfoxide (DMSO) or absolute ethanol, at a final concentration of 10 mM. For *in vitro* treatment, excised strings of follicles were incubated in Grace's insect medium containing 5 μm CD. In *in vivo* experiments, vitellogenic females of *Manduca sexta* were injected in the abdomen with CD at resulting dose 10 μg of CD per g/animal. After 2, 4, 6, 8, 12, 16, 24, and 48 h, the follicles were dissected from female or removed from incubation medium, washed in saline, and fixed immediately for histological analysis, or frozen in liquid nitrogen and stored at -70°C until use for DNA isolation. The CD dose used for injection of *Manduca* adult males was the same as described above for females. Control experiments with *Manduca* adults injected with corresponding doses of pure DMSO or absolute ethanol, or strings of dissected follicles incubated with adequate concentration of either of the solvents in Grace's medium, revealed no detectable effect on the morphology of developing follicles, and no DNA fragmentation was observed under these control conditions.

In the experiments designed to investigate the CD effect in the absence of new RNA or protein synthesis, the CD injection of vitellogenic females was supplemented with actinomycin D or cycloheximide, respectively. The final dose amounted to 10 μg per g/animal for actinomycin D, and 5.62 μg/g of female for cycloheximide. For *in vitro* CD treatment, the Grace's medium contained 5 μg/ml of actinomycin D, or 10 μM cycloheximide, respectively. The doses of actinomycin D and cycloheximide were based on earlier experiments which were shown to inhibit incorporation of tritiated uridine or tyrosine, respectively (Berry *et al.*, 1964; Marek *et al.*, 1988). The control actinomycin D or cycloheximide treatment itself, respectively, did not result in any detectable DNA fragmentation or morphological changes in the cells of developing follicles.

For all CD experiments, freshly emerged *Manduca* adults, as well as adults 1, 2 and 3 days after imaginal ecdysis, were used. All *in vivo* experiments were performed on at least twelve animals. In the case of *in vitro* experiments, a minimum of ten strings of follicles representing single ovarioles dissected from different females were incubated with appropriate medium in separated wells of tissue culture plates. In all repeated experiments, reproducible results were obtained.

Electron microscopy

Adults for indirect flight muscle dissection were injected in the thorax with 4% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After incubation for 2 h at 4°C, the muscle tissue was dissected and fixed by immersion in the same solution overnight at 4°C. Ovarian follicles were excised from CO_2-anaesthetized adult females under saline, immediately transferred to phosphate-buffered glutaraldehyde solution, and fixed overnight at 4°C. The samples were then post-fixed in 1.33% osmium tetroxide in phosphate buffer for 2 h at 4°C, dehydrated, and embedded in Epon 812 resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined with a Zeiss EM10A electron microscope.

Immunohistochemistry and light microscopy

Excised ovarian follicles were fixed in modified Bouin-Hollande fixative (Watson *et al.*, 1993) overnight at 4°C. Standard histochemical techniques were used for sample dehydration, embedding in paraplast, sectioning at 5 µm, deparaffinization, and rehydration. Prior to immunohistochemical staining, slides with sections were blocked with 10% normal goat serum in phosphate-buffered saline containing 0.1% Triton X-100 (PBS-TX) for 30 min at room temperature. Sections were then incubated with primary monoclonal antibody directed against cytoskeletal actin (Amersham, Arlington Height, IL; overnight at 4°C), secondary biotinylated antibody (1 h at room temperature), and, finally, with streptavidin-horseradish peroxidase (HRP; 1 h at room temperature). The enzymatic activity of HRP was detected using N,N'-diaminobenzidine tetrahydrochloride substrate system. Sections were counterstained with Harris hematoxylin to visualize the follicle and nurse cell nuclei. For fluorescence microscopy, Texas red conjugated to streptavidin was used instead of HRP to locate the primary antibody binding. The nuclei were stained specifically for DNA using Hoechst 33342 fluorescent dye. Mounted slides were examined under Zeiss Axioplan microscope equipped with differential interference contrast (Nomarski) optics and epifluorescence.

Isolation of DNA and agarose gel electrophoresis

Frozen follicles for genomic DNA extraction were ground under liquid nitrogen to fine powder. Powdered tissue was resuspended in extraction buffer (100 mM NaCl, 10 mM Tris.Cl pH 8.0, 50 mM EDTA, 0.5% SDS, 100 µg/ml proteinase K) and incubated at 50°C overnight with slow agitation. The samples were then very gently, but thoroughly, extracted twice with phenol, followed by phenol/chloroform, and, finally, chloroform extraction. The DNA was precipitated with absolute ethanol in the presence of ammonium acetate, and resuspended in TE buffer (10 mM Tris.Cl pH 8.0, 1 mM EDTA). Residual RNA was removed by incubating the DNA solution with DNase-free RNase A (1.0 µg/ml) for 3 h at 37°C, followed by organic extraction and ethanol precipitation. Each sample of purified DNA was subjected to electrophoretic separation on 1.6% agarose gel containing 0.5 µg/ml of ethidium bromide.

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