Journal of Cellular Biochemistry 48:373–384 (1992)

The Effect of Age on the Response of the Detrusor to Intracellular Mechanical Stimulus: DNA Replication and the Cell Actin Matrix

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Abstract Benign prostatic hypertrophy and posterior urethral valves present at both extremes of the age spectrum. Both disease processes can obstruct the urinary stream and ultimately have pathophysiological effects on detrusor structure and function. The mechanisms regulating the structural reorganization of the detrusor to a mechanical outflow obstruction are not known. In an attempt to identify maturational differences in myocyte ultrastructure and consequent effects these might have in modifying the response of the detrusor to mechanical stimulus, we studied differences in dynamic nuclear-cytoskeletal interactions in detrusor tissue in an animal model. Using a drug which specifically severs actin, cytochalasin D (CD), as an intracellular mechanical stimulus, we measured changes in nuclear area and the rate of DNA synthesis in detrusor myocytes from young (2–3 week) and old (8–12 mon) guinea pigs. We found that there were age specific differences to intracellular mechanical stimuli in detrusor muscle. Nuclei of myocytes from young animals showed elastic recoil on severing the cell actin matrix and the tissue from young animals increased replicative DNA synthesis with an intracellular stimulus. In contrast, nuclear shape changes in myocytes from old animals suggested less elasticity, and there was no increase in DNA synthesis with disruption of the cell actin matrix. Anti-α-smooth muscle actin antibody and rhodamine phalloidin staining of actin in cytochalasin D treated primary explants of detrusor myocytes showed dose dependent disruption of the actin component of the cytoskeleton.

These results suggest that there are fundamental modifications in detrusor myocyte ultrastructure with age. These maturational changes might result in differences in the pathophysiological and structural reorganization of the detrusor in response to outflow obstruction in infancy and adulthood. Furthermore, they suggest that 1) a tensile equilibrium exists between the myocyte nucleus and cytoskeleton; 2) there appears to be a decrease in myocyte nuclear elasticity with ageing; 3) release of nuclear template restrictions increases activity of DNA polymerase α in young, but not old, detrusor myocytes; and 4) mechanico-chemical signal transduction in detrusor myocytes may be mediated via the cytoskeleton. In addition, based on previous reports of actin within the nucleus, the results suggest that 1) nuclear actin may have a homeostatic structural role, maintaining the tensile equilibrium between nucleus and cytoskeleton, and 2) integrity of nuclear actin may function to maintain the spatial template restriction on DNA polymerase α activity.

Key words: cytochalasin D, DNA synthesis, nuclear matrix, smooth muscle

Benign prostatic hypertrophy and posterior urethral valves present at both extremes of the age spectrum. Both disease processes can obstruct the urinary stream and ultimately have pathophysiological effects on detrusor structure and function. The structural sequelae of bladder outflow obstruction are well recognized with detrusor hypertrophy (Gosling and Dixon, 1983) and connective tissue infiltration (Susset et al., 1978). How the mechanical stimulus of urethral obstruction relates to structural remodelling of the detrusor is poorly understood. It has been suggested that smooth muscle growth may occur as a physiological response to mechanical factors with a fundamental feedback loop relating tensile stretch to tissue growth (Guyton, 1987). Increased mural stress in the obstructed bladder might, therefore, constitute the tensile growth stimulus for detrusor muscle and act through the cell-tissue matrix system of connective tissue, cell adhesion complexes, the cytoskel-

Received November 12, 1991; accepted November 27, 1991.

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eton ultimately affecting the nuclear matrix of the smooth muscle myocyte.

It has been hypothesized that living cells exist under a dynamic equilibrium composed of tension and compression elements within the cytosol (Ingber and Jamieson, 1985; Ingber and Folkman, 1989b). Cytoskeletal actin may constitute tension cables and microtubules, rod-like compression elements, which, when articulated, maintain a *tens*ional int*egrity* or tensegrity (Ingber and Folkman, 1989b). Tensegrity serves to dynamically couple nuclear shape to cell shape changes (Ingber and Jamieson, 1985; Ingber et al., 1987) and it has been suggested that direct mechanico-chemical signal transduction in this cell-tissue matrix system may couple cell shape directly to growth, differentiation, and gene expression (Ingber et al., 1987; Ingber and Folkman, 1989a; Ben-Ze'ev, 1991; Pienta et al., 1991).

We have recently demonstrated that growth of smooth muscle myocytes can respond to mechanical stretch in the absence of other trophic factors (Karim et al., 1991). Since ageing might modify the tensile properties of tissue with increases in smooth muscle collagen (Susset, 1983) or quantitative and qualitative changes in collagen cross-linking (Eyre, 1984), the ability to resist tissue stress might be different to young, immature tissue. How a mechanical stimulus might be interpreted or processed at a cellular level of the detrusor myocyte is not yet understood. In this paper, we have tried to determine how, at an ultrastructural level, ageing might modify the ability of the detrusor myocyte to respond to mechanical stimuli. Our model was guinea pig detrusor muscle tissue from young and old animals stimulated in vitro. Using an intracellular mechanical stimulus by severing the cell actin matrix (Zambetti et al., 1991) we have demonstrated that there are fundamental maturational changes in detrusor myocyte ultrastructure with age. Furthermore, from our results of severing the cell actin matrix with the drug cytochalasin D, a fungal metabolite which specifically disrupts actin by binding actin filament ends (Brown and Spudich, 1981), we suggest that nuclear actin may serve to maintain a tensile equilibrium between nucleus and cytoskeleton. Disruption of nuclear actin may release template restrictions on DNA polymerase α activity and allow replicative DNA synthesis. In addition to our whole tissue preparations, we have documented dose dependent severing of cytoskeletal actin with CD in cell culture of primary explants of detrusor myocytes. These ultrastructural, or cytoskeletal-nuclear, differences we have found between detrusor myocytes from young and old animals might offer an explanation as to why age related differences in the response of the detrusor to mechanical outflow obstruction might be expected.

MATERIALS AND METHODS

Tissue Collection

Young 2–3-week-old, and old, 8–12-month-old, male albino Hartley strain guinea pigs (Charles River) were sacrificed with CO_2 inhalation. Bladders were removed, hemisected in a sagittal plane, and immediately transferred to oxygenated Krebs bicarbonate buffer [composition (mM): NaCl (120), KCl (5.9), $NaHCO_3$ (15.4), $MgCl_2.6H_2$ (1.2), $NaH_2PO_4.2H_2O$ (1.2), $CaCl_2$ (2.5), glucose (11.5)] at 22°C containing penicillin (10 units/ml, Gibco) and streptomycin (10 μg/ml, Gibco).

Detrusor Muscle Strip Preparation

Bladders were pinned to a silicon rubber coated plastic petri dish in Krebs at room temperature. The detrusor was dissected free of mucosa and using translumination, parallel strips (1.5–2 mm in width, 12–18 mm in length) of muscle were prepared using a binocular dissecting microscope. The ends of the strips were then attached to loops of 6–O of surgical silk.

Intracellular Mechanical Stimulation of Myocytes—Severing Cell Actin Matrix

Muscle strips were suspended in a 400 ml beaker containing Krebs and bubbled continuously with 95% O_2 and 5% CO_2 at 37°C. Cytochalasin D (final concentrations of 1×10^{-11}–1×10^{-5} g/ml, CD) was added 10 min prior to the addition of radioactive label. Thymidine,[methyl-^{3}H] (1 μCi/ml, New England Nuclear) was added to the Krebs and the muscle strips allowed to incorporate radioactivity for 6 h. For each experiment control tissue was from the same detrusor, incubated without CD. Cytochalasin D was prepared as stock solutions (1×10^{-8}–1×10^{-2} g/ml) in 100% ethanol and stored at −20°C until used. The vehicle control was 100 μl of 100% ethanol per 100 ml of Krebs solution.

Inhibition of DNA polymerase α by aphidicolin. To determine if the mechanical stimuli

were resulting in replicative DNA synthesis, the experiments were repeated in the presence of aphidicolin. Aphidicolin is a specific inhibitor of DNA polymerase α (Krokan et al., 1979) and, hence, the aphidicolin-sensitive specific activity of DNA (derived from the differences in specific activity of DNA with, and without, aphidicolin) implies replicative DNA synthesis (Watson, 1988). When the experiments were done in the presence of aphidicolin (1 μg/ml, Sigma), the muscle strips were preincubated for 30 min at 37°C prior to the addition of the thymidine, [methyl-^{3}H] and the start of the experimental period. Aphidicolin (1 mg/ml) was prepared as stock solutions in 100% ethanol and stored at −20°C until used. The vehicle control was 1 ml of 100% ethanol per 1 liter of Krebs solution.

Measurement of myocyte nuclear area. Myocyte nuclear area was measured on cytochalasin D treated detrusor muscle tissue to determine the effect of severing the cell actin matrix and quantify the dynamic changes in cytoskeletal-nuclear interactions. A representative strip from each experiment was fixed in formalin (10% neutral buffered) immediately on removing from the radioactive Krebs solution and paraffin-embedded for histology. Hematoxylin and eosin (H&E) stained sections (5 μm) were cut from the paraffin-embedded tissue. Myocyte nuclear area in square microns (μm^2) was measured using a digitizing, nuclear morphometry system (Mohler et al., 1988). Nuclei were viewed (×3,560 magnification) using a Zeiss standard microscope equipped with a Zeiss binocular tube and two ×10 KPL focusable eyepieces. For each slide 80–100 longitudinally or near longitudinally sectioned myocyte nuclei were digitized, on similar fields to those shown in Figure 1.

Assay of thymidine,[methyl-^{3}H] incorporation into DNA. At the end of each experimental period, muscle strips were removed from the radioactive Krebs and the incorporation of thymidine,[methyl-^{3}H] stopped by washing the tissue twice (20 min each wash) in fresh Krebs (4°C) containing cold thymidine (1 mg/ml, Sigma). Muscle strips were then blot dried and frozen in liquid nitrogen for subsequent assay. DNA in the muscle strips was assayed by the diphenylamine reaction (Burton, 1956). Strips were homogenized in 0.4 ml Tris EDTA (100 mM, Sigma). Acid precipitable DNA was coprecipitated with BSA (bovine serum albumin, 1 mg) by PCA (perchloric acid 0.4 M, 4°C). Following centrifugation (4°C, 10,000 rpm, 15 min) the supernate was discarded. The precipitate was resuspended in fresh PCA (0.4 M, 4°C) and the centrifugation repeated. DNA in the precipitate was hydrolysed in PCA (0.8 M, 70°C, 20 min). The DNA hydrolysate was separated following

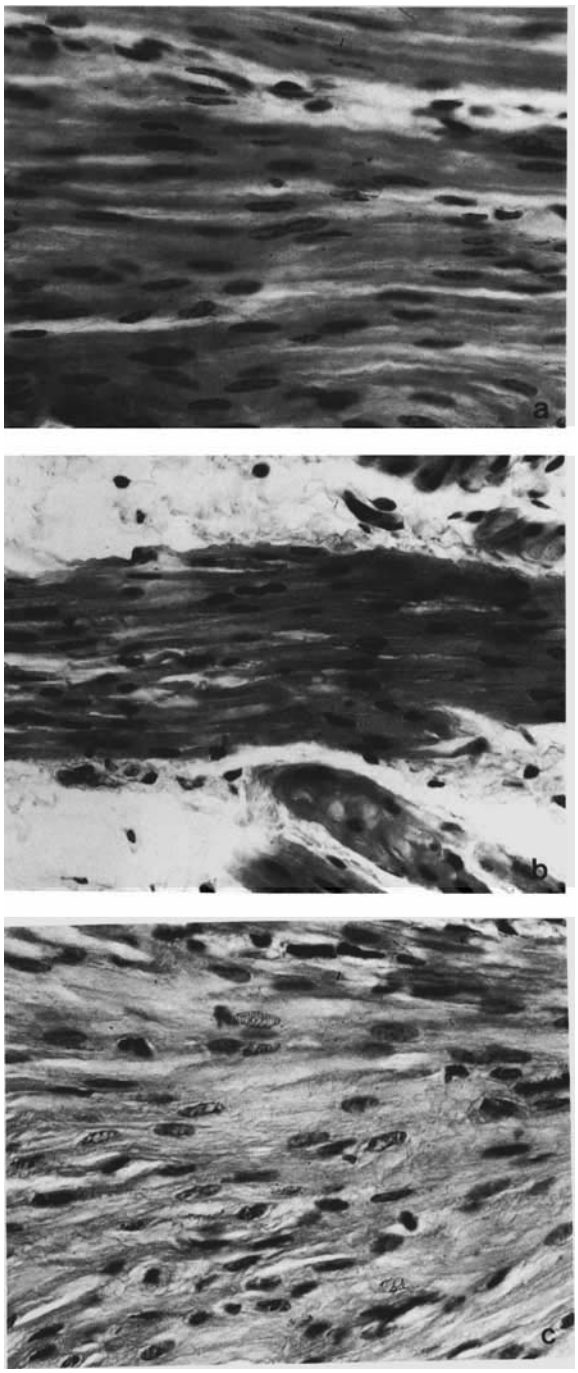

Fig. 1. Hematoxylin and eosin stained longitudinal sections of detrusor muscle from young guinea pig to show the effect of cytochalasin D (CD) on myocyte nuclei. **a:** Control. **b:** CD 1 × 10^{-8} g/ml. **c:** CD 1 × 10^{-5} g/ml. Muscle strips were incubated in Krebs for 6 h in the presence or absence of CD and immediately fixed in formalin. Magnification ×400.

cooling and further centrifugation (4°C, 10,000 rpm, 10 min). The DNA content (μg) in aliquots (100 μl) of the hydrolysate was measured in duplicate, spectrophotometrically at 600 A (Beckman model DU-64 spectrophotometer), against a standard (calf thymus DNA 91.8 μg/ml, Sigma) following reaction in the dark with diphenylamine reagent (20°C, 18 h, composition: 100 ml glacial acetic acid, 1.5 ml concentrated sulfuric acid, 1.5 g diphenylamine, Sigma, and 0.5 ml acetaldehyde 1.6%, Sigma). Thymidine,[methyl-^{3}H] incorporation into DNA was measured in duplicate on aliquots (100 μl) of DNA hydrolysate in scintillation fluid (5.0 ml, Aquasol-2, Dupont). The specific activity of DNA in each muscle strip was subsequently expressed as counts per minute (CPM) per μg DNA. The specific activity of DNA in each muscle strip was then expressed as a percentage of its respective control group, taken from the same detrusor. Thus, specific activity of DNA was 100% in the control group and the relative specific activity of DNA was obtained for each cytochalasin D treatment group.

Autoradiography of in vitro thymidine,[methyl-^{3}H] labeled muscle tissue. Autoradiography was performed on the muscle strips to identify the detrusor cell population undergoing DNA synthesis. Muscle strips from control tissue were fixed for a minimum of 24 h in 10% buffered formalin. Sections (5 μm) were mounted on microscope slides and coated with NTB2 photographic emulsion (dilution 1:1 with water, Eastman Kodak). The emulsion was exposed to the tissue sections in light-tight slide boxes with desiccant (Humicaps, United Desiccants—Gates) at 4°C for 1 to 3 days. Following exposure the emulsion was developed in Dektol (1:1 dilution with water for 2 min, 17°C, Eastman Kodak), washed in water (15 sec at 17°C), fixed in Kodak Rapid Fixer (3 min, 17°C), and washed in running tap water (45 min, 18°C). The slides were counter stained with 0.1% toluidine blue in 1% sodium borate (pH 9.2, 90 sec) and air dried for 18 h prior to coverslipping. A representative section from each muscle strip was cut for hematoxylin and eosin staining.

Cell Culture of Detrusor Cells to Demonstrate Disruption of Cell Actin Matrix

Preparation of primary detrusor explants. Primary cultures of detrusor myocytes were established to visually document the dose related effects of severing the cell actin matrix with cytochalasin D. Mucosa free detrusor was prepared as above in Hank's Balanced Salt Solution (HBSS) with Ca^{2+} and Mg^{2+} (Gibco) containing penicillin (10 units/ml, Gibco) and streptomycin (10 μg/ml, Gibco). Following dissection the muscle tissue was sterilized briefly in 70% ethanol and washed in fresh HBSS. The muscle tissue was minced into small cubes (≈ 1 mm^3) and digested in fresh HBSS containing collagenase (1 mg/ml, Gibco) in a plastic culture tube for 8 h at 37°C. Dispersal of the cells was aided by gentle pipetting up and down of the debris at the end of the period of digestion. Cells were washed in triplicate (HBSS) and resuspended in 2.0 ml of Dulbecco's modified Eagle's medium (DMEM). Aliquots of the cell suspension (0.1 ml) were plated onto single well Chamber Slides™ (Lab-Tek®, Nunc) which were precoated (5–10 μg/cm^2) with E-C-L (entactin-collagen IV-laminin) Cell Attachment Matrix (Upstate Biotechnology). The cell culture medium was DMEM supplemented with 10% fetal bovine serum (Gibco) and was changed twice weekly. Cultures were maintained in a humidified atmosphere at 37°C with 5% CO_2.

Disruption of cellular actin in cultured cells with cytochalasin D. After 10–12 days growth in cell culture, the primary explants of detrusor cells were treated with cytochalasin D. Cytochalasin D (1×10^{-8}, 1×10^{-7}, and 1×10^{-5} g/ml) was prepared in fresh culture medium from stock solutions, prewarmed, and substituted for established culture medium. The vehicle control was 100% ethanol (1 μl/1 ml). Cells were incubated with the new medium for a further 6 h, washed briefly in phosphate-buffered saline (PBS) ×2 and fixed in 3.75% formaldehyde/PBS for 10 min at room temperature.

Fluorescent staining for cytoskeletal actin. Fixed cells were rewashed in PBS ×2 and extracted with acetone at −20°C for 5 min. Cells were incubated (1 h, 22°C) with a primary antibody, a smooth muscle specific mouse monoclonal anti-α-smooth muscle actin (Skalli et al., 1986) (Sigma), at a dilution of 1:400. The secondary antibody was an FITC-conjugated goat anti-mouse IgG (Sigma) diluted 1:128 (45 min, 22°C). Slides were washed in PBS ×2 prior to mounting with PBS/glycerol (1:1) and coverslipping. Coverslips were sealed with clear nail polish. Certain slides, in addition, were prepared for double fluorescent staining with rhodamine phalloidin. In this case, prior to adding mounting medium, slides were stained (20 min) with 10 μl

of rhodamine phalloidin (3.3 M in methanol, Molecular Probes), evaporated, and reconstituted in 400 μl PBS. Slides were then rewashed briefly (PBS ×2) and mounted as described above. FITC fluorescesence was visualized under blue light (λ = 436–460 nm) and rhodamine phalloidin fluorescesence visualized under green light (λ = 530–585 nm) using a Zeiss photomicroscope. Pictures were taken on P800-1600 Extachrome film (Kodak) through a ×63 objective.

Statistical Analysis

Values presented in the results are mean for each group ± standard error of the mean (SEM). Comparison for statistically significant differences between multiple groups was determined following analysis of variance and a Tukey multiple comparisons test (Statsgraphics v4.0, Statistical Graphics Corporation). When indicated, data was initially ranked and rank values used for statistical analysis (Conover and Iman, 1981). A probability of $P < 0.05$ was considered significant.

RESULTS

Effect of Disrupting the Cell Actin Matrix—An Intracellular Mechanical Stimulus

There were age specific, qualitative and quantitative, differences in myocyte nuclei on severing the cell actin matrix.

Qualitative and quantative changes in myocyte nuclei. Representative hematoxylin and eosin stained sections taken from the same detrusor muscle to demonstrate the effect of cytochalasin D on myocyte nuclei in tissue from a young guinea pig are shown (Fig. 1). Low dose cytochalasin D ([CD] L, 1×10^{-8} g/ml) resulted in small, darkly staining myocyte nuclei (Fig. 1b) which differed from the appearance of nuclei incubated in Krebs alone (CONTROL) (Fig. 1a). In the presence of high dose cytochalasin D ([CD] H, 1×10^{-5} g/ml), myocyte nuclei appeared larger and paler compared to those in low dose cytochalasin D (Fig. 1c). In detrusor tissue incubated in [CD] H in addition to aphidicolin (1 μg/ml, [CD] H+APD), nuclei were similar in appearance and size to those in [CD] H alone (not shown). Myocyte nuclei in detrusor muscle tissue from old animals treated in [CD] H and [CD] H+APD were similar to the nuclei in the young tissue, in that they appeared larger and paler than those in low dose cytochalasin D (not shown). In low dose cytochalasin D, however, nuclei in detrusor tissue from old animals did not appear to differ from the CONTROL group and did not appear small and dark as did the comparable low dose cytochalasin D group for the young animals (not shown).

In young guinea pig detrusor tissue, myocyte nuclear area was significantly less in the low dose cytochalasin D group compared to the CONTROL group (CONTROL = 16.2 ± 0.25 μm^2 vs. [CD] L = 12.6 ± 0.22 μm^2, $P < 0.01$). In tissue from the same animals incubated with both [CD] H and [CD] H+APD, nuclear area was significantly greater than CONTROL ([CD] H = 18.7 ± 0.26 μm^2, $P < 0.01$ and [CD] H+APD = 17.7 ± 0.23 μm^2, $P < 0.01$) (Fig. 2a).

In tissue from old guinea pigs, area of myocyte nuclei in the low dose cytochalasin D group was no different to the CONTROL group (CONTROL = 15.1 ± 0.27 μm^2, [CD] L = 15.5 ± 0.22 μm^2). In tissue from the same old guinea pigs nuclear area was significantly greater than CONTROL in both the [CD] H and [CD] H+APD groups ([CD] H = 19.7 ± 0.34 μm^2, $P < 0.01$ and [CD] H+APD = 21.6 ± 0.31 μm^2, $P < 0.01$) (Fig. 2b).

Effect of intracellular mechanical stimulus on the rate of DNA synthesis in detrusor muscle. In addition to the histological differences, there were dose and age specific effects of cytochalasin D on the rate of DNA synthesis in guinea pig detrusor muscle tissue. Cytochalasin D affected the rate of DNA synthesis *in* detrusor muscle from young guinea pigs in a dose dependent manner. In detrusor muscle tissue from young animals, lower doses of cytochalasin D ($\leq 1 \times 10^{-7}$ g/ml) did not affect the relative specific activity of DNA significantly. Higher doses of cytochalasin D ($\geq 1 \times 10^{-6}$), however, increased the relative specific activity of DNA significantly compared to the CONTROL group ($P < 0.01$). Of the concentrations of cytochalasin D used, the highest concentration (1×10^{-5} g/ml, [CD] H) resulted in the greatest increase in relative specific activity of DNA. In CONTROL tissue, aphidicolin decreased the relative specific activity of DNA in detrusor muscle strips to 25.2 ± 2.6%. In [CD] H, aphidicolin decreased the relative specific activity of DNA from 197.5 ± 27.7% to 26.4 ± 1.8% (Fig. 3a). Thus, the aphidicolin sensitive relative specific activity of DNA was 74.8 ± 6.6% in control tissue and 171.1 ± 27.7% in [CD] H treated tissue. [CD] H, therefore, doubled the rate of replicative DNA synthe-

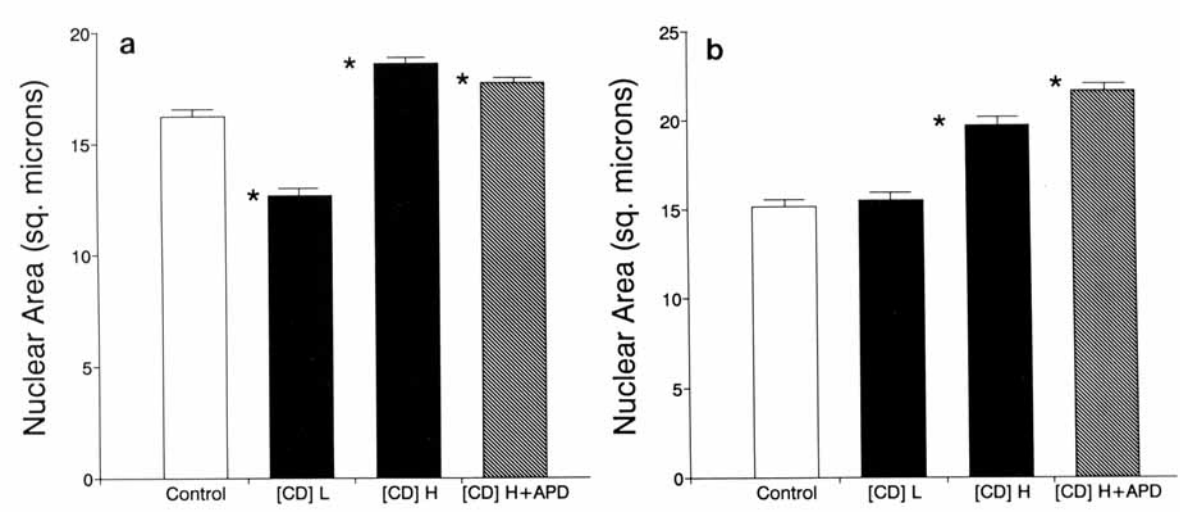

Fig. 2. Nuclear area of myocytes in detrusor muscle strips from (**a**) young and (**b**) old guinea pigs in the presence and absence of cytochalasin D (CD). Control, no CD; [CD] L, Low dose CD (1×10^{-8} g/ml); [CD] H, High dose CD (1×10^{-5} g/ml); [CD] H+APD, High dose CD (1×10^{-5} g/ml) and aphidicolin (1 μg/ml). Nuclear area in square microns (μm^2) was measured as described in Materials and Methods (n = 180–280). *$P < 0.01$ compared to control.

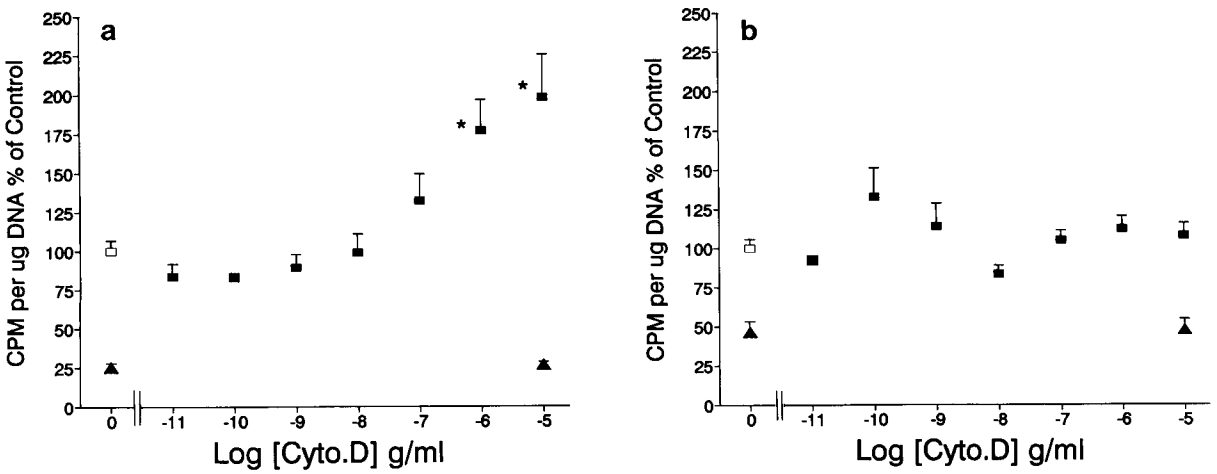

Fig. 3. Effect of cytochalasin D (CD) of the rate of DNA synthesis in detrusor muscle from (**a**) young and (**b**) old guinea pigs. The relative specific activity of DNA in control (100%) and experimental groups is plotted as counts per minute per microgram DNA as percent of control tissue taken from the same detrusor (CPM per μg DNA % Control). Detrusor muscle strips were incubated in Krebs with and thymidine,[methyl-^{3}H] (1 μC1/ml) and in the (□) absence (Control) or (■) presence of CD. Control and high dose CD (1×10^{-5} g/ml) were, in addition, incubated without and (▲) with aphidicolin (1 μg/ml) (n = 5–20) *$P < 0.01$.

sis in detrusor muscle tissue from young guinea pigs.

In contrast to its effects on tissue from young animals, cytochalasin D did not increase the rate of DNA synthesis in detrusor muscle tissue from old animals (Fig. 3b). In CONTROL tissue, aphidicolin decreased the relative specific activity of DNA to 46 ± 6.6%. In [CD] H, aphidicolin decreased the relative specific activity of DNA from 108.1 ± 7.9% to 47.7 ± 6.7% (Fig. 3a). Thus, for old animals, the aphidicolin sensitive relative specific activity of DNA was 53.4 ± 6.3% in CONTROL tissue and 60.4 ± 7.9% in [CD] H treated tissue. [CD] H, therefore, did not change the rate of replicative DNA synthesis in detrusor muscle tissue from old guinea pigs.

Autoradiography. Autoradiography of detrusor muscle strips in tissue from young guinea pigs showed silver grains over myocyte nuclei (Fig. 4a). There was an essential absence of

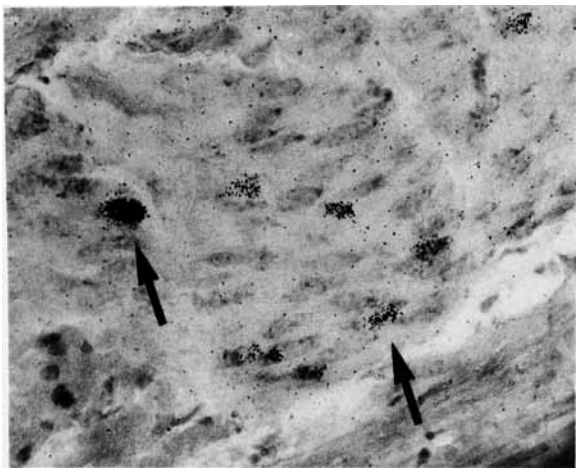

Fig. 4. Autoradiography on histological section of in vitro thymidine,[methyl-^{3}H] labeled detrusor muscle strip stained with toluidine blue stained to demonstrate (arrows) myocyte DNA synthesis. Magnification ×600.

grains over nonmuscle cells, implying myocytes were the predominant detrusor cell population undergoing DNA synthesis. Muscle strips from old guinea pigs were qualitatively similar, but quantitatively differed with fewer labeled nuclei (not shown).

Appearance of Myocyte Cell Actin Matrix Disrupted by Cytochalasin D

Actin fibers in control cells stained with rhodamine phalloidin were clearly visible as a network of filaments throughout the cytosol (Fig. 5a). Using double fluorescesence with the smooth muscle specific mouse monoclonal anti-α-smooth muscle actin, an identical pattern of filaments was seen, implying 1) the mouse antibody recognized guinea pig smooth muscle actin, and 2) we had successfully established primary cultures of smooth muscle myocytes. The smooth muscle specific antibody was used for subsequent visualization of myocyte cytoskeletal actin. There was a dose related effect of cytochalasin D on the pattern of the cytoskeletal actin. The lowest concentration of cytochalasin D used (1×10^{-8} g/ml) resulted in little visible change in the actin filaments, but a decrease in nuclear area was suggested (Fig. 5d). With increasing concentrations, the visible effect of cytochalasin D on the actin filament was more apparent (Fig. 5e). The highest dose of cytochalasin D (1×10^{-5} g/ml) resulted in marked disorganization of the actin filaments, but the nucleus was still intact (Fig. 5f).

DISCUSSION

In this paper we have found structural and functional differences in the response of detrusor myocytes from young and old guinea pigs to intracellular mechanical stimuli. In addition, our data demonstrates that an intracellular mechanical stimulus alone can directly modulate DNA synthesis in young detrusor myocytes in vitro. Our ability to demonstrate dissimilar responses of nuclei in young and old tissue to disrupting the cell actin matrix suggests there might be fundamental maturational differences in detrusor myocyte ultrastructure and cytoskeletal-nuclear interactions with age. In addition, nuclear enlargement in the presence of high dose cytochalasin D suggests that actin may have a homeostatic structural role not only in the cytoplasm, but within the eukaryotic cell nucleus, maintaining a dynamic tensile equilibrium between nucleus and cytosol. Furthermore, an increase in replicative DNA synthesis with nuclear enlargement, which in turn was independent of inhibition of DNA polymerase α activity by aphidicolin, suggests nuclear actin may function to maintain a spatial template restriction on replication of the genome.

The advantages of using whole tissue preparations of detrusor muscle were twofold. First, detrusor muscle taken directly young and old guinea pigs gave us a population of young and naturally aged cells respectively, in contrast to cultured cells where serial passage might alter phenotype and aging characteristics. Second, in anchorage dependent cell growth, there is a tendency for cell and nucleus to flatten out. Morphology of cultured cells, therefore, clearly focusses on a two-dimensional plane. In cultured cells a decrease in nuclear area might be readily apparent on severing cytoskeletal actin and nuclei adopting a more spherical shape. Subsequent nuclear swelling, however, might not be documented as easily as with whole tissue preparations where the passive (or active) support by components of the cytosol, in all three dimensions, might make the nuclear shape changes more physiological.

The interpretation of our experimental data and how it might relate to the basic mechanisms of cell growth must be viewed in light of current concepts of DNA organization within the eukaryotic nucleus. Specific attachment sites of DNA to the nuclear matrix are thought to organize

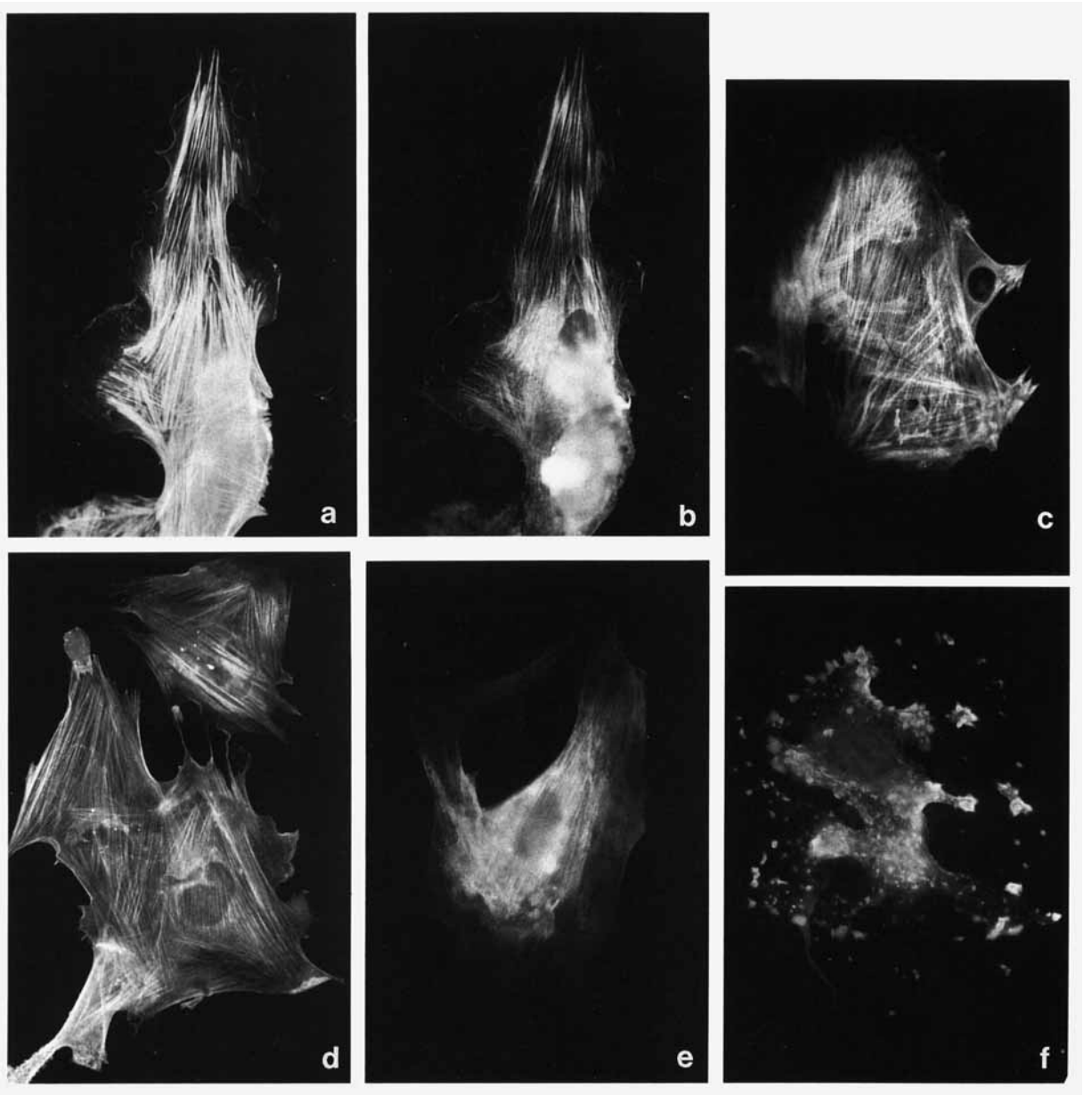

Fig. 5. Primary cell culture of detrusor myocytes showing effects of increasing concentration of cytochalasin D (CD) on cytoskeletal actin. Stained with **(a)** rhodamine phalloidin and **(b, c, d, e, f)** indirect immunofluorescence using a smooth muscle specific mouse monoclonal anti-α-smooth muscle actin (primary antibody) and an FITC-conjugated goat anti-mouse IgG (secondary antibody). a, b, c: Control. (a) and (b) are the same cell, (a) being a positive control for (b) and actin. d: CD 1×10^{-8} g/ml. e: CD 1×10^{-7} g/ml; f: CD 1×10^{-5} g/ml. Cells were cultured and treated as described in Materials and Methods.

the genome within the interphase nucleus (Pienta et al., 1989). The nuclear matrix is, in turn, the *residual structural framework of the cell nucleus* (Berezney and Coffey, 1975). Newly synthesized DNA is associated with the nuclear matrix (Berezney and Coffey, 1975) and it has been suggested that there are fixed sites of DNA replication where replication complexes are attached to the nuclear matrix proteins (Jackson and Cook, 1986). Whether the attachment sites of DNA to the nuclear matrix during interphase and 'S' phase are the same is not known but there appears to be specific spatial organization of the eukaryotic nucleus (Spector, 1990). In 1963, Cairns predicted that replication of prokaryotic circular DNA demanded a complex that could act as a swivel (Cairns, 1963) and subsequently it has been shown that DNA topoi-

somerase II is associated with the replicating complex in eukaryotic cells (Nelson et al., 1986). There are, thus, immense topographical considerations for replication of the genome and an increase in nuclear size appears to be a prerequisite for nucleic acid synthesis (Graham et al., 1966; Nicolini et al., 1986). Direct evidence for release of template restriction on exogenous DNA polymerase activity was demonstrated in isolated rat liver nuclei induced to swell by acidic polymers (Coffey et al., 1974). Further evidence that nuclear size might be important to DNA synthesis in intact cells was presented by Ingber et al. (1987), who, in addition, showed that nuclear area of capillary endothelial cells could be determined by modulating interactions with the extracellular matrix and, thus, implied cell-nuclear shape changes might be coupled via the cytoskeleton (Ingber et al., 1987). Subsequently it has been suggested that the mechanical constraints of a compact nucleus might be modified by cytoskeletal-nuclear interactions, thereby directly modulating DNA synthesis or specific gene expression (Ingber and Folkman, 1989a). The existence of tensegrity and evidence of dynamic nuclear-cytoplasmic interactions suggests that there must be homeostatic structural proteins within the nucleus, maintaining the internal milieu.

In this study we have focused on an intracellular mechanical stimulus as a modulator of growth for detrusor myocytes. Our investigation of actin and the possible effects maturation might have in changing its cytoskeletal-nuclear interactions in detrusor myocytes have shed new light on potential functions of the cell actin matrix. Actin is ubiquitous in eukaryotic cells and in a polymerized state (F-actin), it is a cytoskeletal microfilament (Ingber and Folkman, 1989b). Various naturally occurring actin binding proteins (e.g., gelsolin, profillin) interact with actin and regulate cytoskeletal microfilament assembly (Ingber and Folkman, 1989b). Though F-actin has been shown to be part of the nuclear matrix (Nakayasu and Ueda, 1983), little attention has been paid to its potential homeostatic structural role in maintaining the integrity of the cell nucleus. Multiple forms of actin have been identified in eukaryotic cells (Garrels and Gibson, 1976) and it has been reported that nuclear actin, though similar, is not identical to cytoplasmic actin (Bremer et al., 1981). There might, thus, be potential differences in sensitivity to severing of nuclear and cytoplasmic actin by cytochalasin D. Our data, suggests that low dose cytochalasin D might preferentially sever cytoskeletal actin and subsequently high dose cytochalasin D disrupt nuclear actin,, resulting in nuclear swelling.

The effects of cytochalasin D on myocyte nuclei in detrusor tissue from young animals might best be described as biphasic. Cytochalasin D in low concentration decreased and in high concentration increased nuclear size. These results might be explicable if we assume differential sensitivity of cytoskeletal and nuclear actin to cytochalasin D and the existence of a cytoskeletal-nuclear tensile equilibrium. Low dose cytochalasin D appeared to produce minimal changes in the pattern of cytoskeletal actin in the cultured cells, but this was obviously sufficient to upset the tensile equilibrium between cytoskeleton and nucleus, resulting in nuclei "shrinking" both in cultured myocytes and our preparations of young detrusor muscle tissue. High dose cytochalasin D ([CD] H) might be in adequate concentration to sever nuclear actin and, thus, result in nuclear swelling. This explanation for the nuclear shape changes we demonstrated is shown schematically (Fig. 6). How nuclear enlargement might be brought about is not yet clear, but there might be residual intact cytoskeletal structures (e.g., intermediate filaments of microtubules) which together might "pull" on an atonic nucleus. Nuclear swelling might, consequently, be passive, active, or a combination of both. Further studies, using anti-microtubule or anti-intermediate filament agents, might help clarify this. The thesis that nuclear actin might have a homeostatic structural role is further supported by the recent discovery of Mbh1 (*myc-b*asic motif *h*omolog-1) a gelsolin/severin-related protein. Mbh1 was reported to localize to fibroblast nuclei and it has been suggested that it might regulate cytoplasmic and/or nuclear architecture through interactions with actin (Prendergast and Ziff, 1991).

Disruption of nuclear actin by high concentrations of cytochalasin D, of course, may not be the only explanation for the nuclear enlargement we demonstrated. It is possible that high dose cytochalasin D might have other nonspecific effects on cell structure or metabolism. It is well known that the cytochalasins can inhibit a variety of biological functions: glucose transport, thyroid secretion, growth hormone release, platelet aggregation, and clot contraction (Budavari et al., 1989). Which of these might be

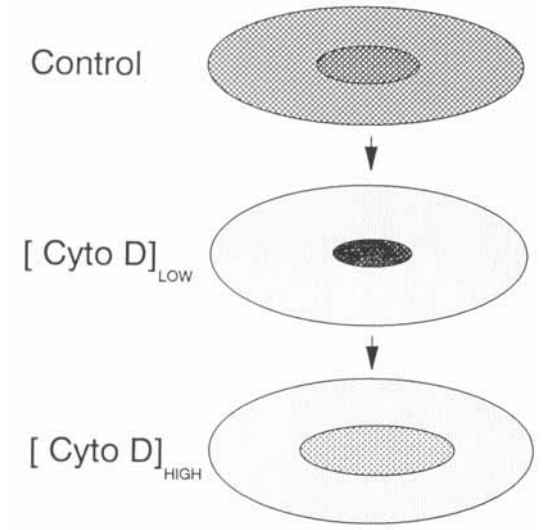

Fig. 6. Schematic showing possible effect of cytochalasin D on cell actin matrix. Differential sensitivity of cytoskeletal and nuclear actin may explain the observed changes in myocyte nuclear area in detrusor muscle tissue from young animals. With a low concentration of cytochalasin D ($[Cyto\ D]_{LOW}$, 1×10^{-8} g/ml), cytoskeletal actin is severed. Nuclear area decreases and intensity of staining increases as nucleus condenses on disrupting the putative tensile equilibrium between nucleus and cytoskeleton. A high concentration of cytochalasin D ($[Cyto\ D]_{HIGH}$, 1×10^{-5} g/ml) may sever nuclear actin and allow nuclear swelling either passively or actively. The cell actin matrix in smooth muscle myocytes from old animals might respond similarly, but the lack of decrease in nuclear area with $[Cyto\ D]_{LOW}$ suggests less nuclear elasticity with ageing (see Discussion for explanation).

related to their effects on the cytoskeleton is not known, but there is certainly scope for nonspecific metabolic effects in high dosages. The maximum dose of cytochalasin D we used (1×10^{-5} g/ml) has previously been reported to induce *c-fos* expression in cultured cells (Zambetti et al., 1991). In this case it was suggested that the chemo-mechanical stimulus of disrupting the cytoskeletal actin resulted in specific gene expression. The maximum dose we used is, therefore, not without precedent and, furthermore, might not be lethally toxic to the cell.

The ability of myocyte nuclei in young tissue to "shrink" on treatment with low dose cytochalasin D suggests that there is indeed a tensile equilibrium between the nucleus and cytoskeleton. In addition, it might imply that young nuclei have plasticity or elasticity. The myocyte nuclei in older tissue, in contrast, did not decrease in area with low dose cytochalasin D. This, though indirect evidence, suggests old nuclei might either 1) have less elasticity or be more "rigid" compared to young nuclei, or 2) be under a lesser degree of tension with the cytoskeleton. Maturation might, therefore, result in fundamental reorganization of the nuclear ultrastructure. On the other hand, nuclear enlargement with high dose cytochalasin D in detrusor muscle from old guinea pigs suggests that the same basic mechanism inducing nuclear swelling might be implicated in both young and old tissue.

Our observations that the cytochalasins can affect the rate of DNA synthesis in cells are not original. Several previous investigators have used the cytochalasins to either stimulate (Rothberg et al., 1978) or inhibit (Maness and Walsh, 1982) DNA synthesis. Rothberg et al. (1978), using cytochalasin B on whole tissue preparations of epidermal and dermal cells from 40–70-day-old mice, found very similar dose dependent stimulatory effects on DNA synthesis to those we have reported for young detrusor muscle. Though they demonstrated DNA synthesis in the basal cells by autoradiography, they did not comment on changes in nuclear morphology. In cultured cells, initiation of DNA synthesis by combinations of serum, epidermal growth factor, fibroblast growth factor, and insulin could be irreversible blocked by low dose cytochalasin B. The inhibitory effect of the low dose cytochalasin B on initiation of DNA synthesis correlated with disorganization of the cytoskeleton actin microfilaments. This observation, though in cell culture, is of particular interest to our model, since it suggests that tension on the nucleus through the cytoskeleton maintains a spatial nuclear geometry necessary for DNA synthesis. Our observations that in young tissue an increase in DNA synthesis accompanied the increase in nuclear size further supports the concept that nuclear geometry can modulate the rate of DNA synthesis. Furthermore, myocyte nuclear enlargement by high dose cytochalasin D in young tissue despite inhibition of DNA polymerase α activity by aphidicolin suggests that the spatial template restriction might determine the rate of DNA synthesis, rather than the rate of DNA synthesis determining nuclear size.

DNA synthesis in eukaryotic cells is proving to be increasingly complex and may involve up to four different polymerases, polymerase α (primase activity), polymerase β (repair), polymerase δ (lagging strand extension), polymerase ϵ (leading strand extension), in addition to telomerase (Linn, 1991). Though the various DNA polymerases may not function similarly within

different eukaryotic organisms or cell types, polymerase α activity is ubiquitous in eukaryotic cells (Linn, 1991). In our experiment we have not sought to completely abolish DNA polymerase α activity by aphidicolin, but rather to demonstrate differences in the aphidicolin-sensitive specific activity of DNA in young and old detrusor muscle, with and without high dose cytochalasin D, using the same dose of aphidicolin (1 μg/ml). We chose this dose of aphidicolin as it has been shown to inhibit over 90% of DNA polymerase α activity in intact HeLa cells (Krokan et al., 1979). DNA synthesis in the presence of aphidicolin implies either 1) aphidicolin-resistant DNA synthesis, or 2) the inhibitory effect of aphidicolin is decreasing with time. Aphidicolin-resistant DNA synthesis may be either 1) noneucaryotic DNA synthesis (Krokan et al., 1979), which is unlikely to account for differences between control and tissue under tension, since both were in the same organ bath in the presence of antibiotics, 2) mitochondrial DNA synthesis, which only comprises approximately 2% of total cellular DNA and is dependent on DNA polymerase γ activity (Watson, 1988) (since autoradiography showed the thymidine,[methyl-^{3}H]- activity to be located over smooth muscle nuclei, mitochondrial DNA synthesis is unlikely to account for significant DNA synthesis in our experiment), or 3) DNA repair, dependent on DNA polymerase β activity (Watson, 1988). The inhibitory effect of aphidicolin is unlikely to be decreasing with time since control tissue from young bladder muscle showed no difference in DNA synthesis rate in the presence of aphidicolin after 6 or 12 h incubation.

The maturational differences we clearly demonstrated in response to an intracellular chemomechanical stimulus of detrusor muscle in young and old guinea pig tissue will be of particular interest to bladder muscle physiologists. It has previously been reported that myocyte hypertrophy is the response of the adult detrusor to outflow obstruction secondary to benign prostatic hyperplasia (Gosling and Dixon, 1983). Brent and Stephens (1975) demonstrated experimentally that there were maturational differences in the myocyte response to a bladder outflow obstruction in the rabbit. In young animals, myocyte hyperplasia was the predominant early effect of obstruction whereas in adult animals, myocyte hypertrophy preceded a lesser degree of hyperplasia. The mechanisms underlying these observations were not known. Our experimental data is, thus, of particular relevance in that at a more fundamental level the maturational effects on cytoskeletal-nuclear interactions might offer an explanation as to why age related differences in the response of the detrusor to outflow obstruction might be expected. The lack of increase in replicative DNA synthesis in detrusor muscle tissue from old guinea pigs by severing the cell actin matrix might be explained by an age related decrease in DNA polymerase α activity as has been reported for other tissue (Mozzhukhina et al., 1991).

In conclusion, we have shown through severing the cell actin matrix and initiating an intracellular mechanical stimulus that there are fundamental differences in detrusor myocyte ultrastructure and function with ageing. Furthermore, our results suggest that 1) a tensile equilibrium exists between the myocyte nucleus and cytoskeleton, 2) there appears to be decrease in myocyte nuclear elasticity with ageing, and 3) release of nuclear template restrictions increases activity of endogenous DNA polymerase α in young, but not old, detrusor myocytes. In addition, based on previous reports of actin within the nucleus, the results suggest that 1) nuclear actin may have a homeostatic structural role, maintaining the tensile equilibrium between nucleus and cytoskeleton, and 2) integrity of nuclear actin may function to maintain the spatial template restriction on DNA polymerase α activity. These observations, though, from experiments on normal detrusor muscle may have considerable implications not only for our understanding of bladder muscle pathophysiology, but for cell and tumour biology as well.

ACKNOWLEDGMENTS

This work was supported by PHS grants D538466-03 and AM19300. Mr. Omer Karim was supported by a King Edward VII Hospital Fund Travel Bursary and an Ethicon Foundation Travel grant from the Royal College of Surgeons of Edinburgh. This paper is part of work to be submitted for the award of a higher degree in the Faculty of Medicine from the University of London.

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