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Influence of the α_1-Adrenergic Antagonist, Doxazosin, on Noradrenaline-Induced Modulation of Cytoskeletal Proteins in Cultured Hyperplastic Prostatic Stromal Cells

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BACKGROUND. Doxazosin, an α_1-adrenergic antagonist, inhibits sympathetic contraction of prostatic stromal smooth muscle cells and is used in the relief of obstructive benign prostatic hyperplasia (BPH). In vitro application of noradrenaline stimulates expression of cytoskeletal filaments, particularly actin and myosin, by prostatic stromal cells, thus enhancing their differentiation towards smooth muscle cells. This study examined the possible role of doxazosin in reversing this phenotypic modulation as well as in inhibiting smooth muscle cell contraction.

METHODS. Stromal cell tissue cultures derived from 10 human hyperplastic prostates were rendered quiescent by reduction of stripped fetal calf serum (FCS) to 1% (v/v) in the medium followed by treatment with 20 μM noradrenaline and/or 1 μM doxazosin for 10 days. Doxazosin, in 10-fold increments of concentration, was also added, separately, to two of these cell cultures, which were either quiescent or growing in 10% normal (unstripped) FCS. Harvested cells were labelled with fluorescein-labelled antisera to smooth muscle cytoskeletal filaments, and their individual fluorescence levels were analyzed flow-cytometrically.

RESULTS. Noradrenaline increased expression of all cytoskeletal filaments studied. This effect was greatest for actin and myosin in proliferating cell cultures. Doxazosin largely reversed the increase in filament expression. This effect was most significant for actin and myosin and greatest in quiescent cultures. However, inhibition of the agonist effect of noradrenaline by doxazosin showed no clear dose-related response, in that expression of cytoskeletal filaments was differentially inhibited.

CONCLUSIONS. The data suggest that doxazosin may inhibit not only stromal contraction of differentiated smooth muscle cells in BPH but also the phenotypic modulation of stromal smooth muscle cell differentiation induced by noradrenaline. These actions, together, may render prostatic stroma less contractile, and hence less able to respond to sympathetic stimulation, in patients with BPH. While effects on isolated stromal cells are of undoubted importance, failure to demonstrate a consistent dose-response relationship between expression of smooth muscle cell phenotype and inhibition by doxazosin suggests that additional influences, including humoral factors as well as the proximity of differentiated epithelium, are also

Abbreviations: FITC, fluorescein isothiocyanate; BPH, benign prostatic hyperplasia; FCS, fetal calf serum.

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likely to be involved in this interaction in the intact tissue. *Prostate 38:216–227, 1999.*

KEY WORDS: **human prostate; stroma; smooth muscle cells; tissue culture; flow cytometry; noradrenaline; doxazosin**

INTRODUCTION

α_1-adrenergic receptor-blocking agents are now commonly employed to relieve urethral obstruction in benign prostatic hyperplasia (BPH). The rationale behind this treatment is that stimulation of prostatic tissues by sympathetic nerves induces contraction of smooth muscle cells in the stroma [1]. A considerable proportion of the hyperplastic prostate is occupied by stroma [2–5], of which approximately one half comprises cells of differentiated smooth muscle phenotype [6,7]. Noradrenaline appears to act on these stromal smooth muscle cells by binding to plasma membrane α_{1A}-adrenoceptors [1,8–12]. Thereafter, ensuing contraction of stromal smooth muscle cells exacerbates any passive obstruction to urethral outflow.

The α_1-adrenoreceptor antagonist, doxazosin (Cardura®, Pfizer, Sandwich, Kent, UK), originally introduced to treat systemic hypertension, has recently been used in the management of BPH. Clinical trials have demonstrated that long-term administration of doxazosin significantly increases both the maximum and mean rates of urinary flow in men with BPH [1,13–16]. The agent is well-tolerated by both normotensive and hypertensive patients [15].

Recently, we demonstrated that noradrenaline, at concentrations above 10 μM, enhances immunoexpression of several cytoskeletal filament proteins in human prostatic stromal cells grown in tissue culture [17]. Immunofluorescence of α-smooth muscle actin, myosin, desmin, and talin all increased progressively with elevating concentrations of noradrenaline, as assessed by flow cytometry. This phenotypic modulation was considerably greater when the cells were plated sparsely and simultaneously stimulated by addition of 10% fetal calf serum (FCS) to the culture medium.

The present investigation was undertaken to determine whether the blockade of α_1-adrenoceptors by doxazosin prevents modulation of smooth muscle cell differentiation induced by noradrenaline, as determined by enhanced expression of these cytoskeletal proteins. Accordingly, sparsely-plated prostatic stromal cells were grown in tissue culture as described in the previous study [17], and were then exposed to noradrenaline in combination with various concentrations of doxazosin. Since sparsely-plated and rapidly-proliferating cells do not accurately reflect the situation in the intact, hyperplastic prostatic stroma [17], simultaneous experiments were performed to examine the effects of doxazosin on confluent cells which were growth-inhibited by a supplement of only 1% (v/v) dialyzed and carbon-stripped serum. By analyzing cultures of prostatic stromal cells in early passage grown under these conditions, the objective of these studies was to determine whether doxazosin can modify the phenotype of stromal cells in hyperplastic prostates stimulated by noradrenaline and thereafter to establish the concentrations of antagonist required to induce the effect in this model system.

MATERIALS AND METHODS

Isolation of Stromal Cells

Prostatic tissues were obtained from patients who had undergone transurethral prostatic resection to alleviate symptomatic BPH. For these studies, prostatic samples were taken only from those cases in which simultaneous histopathological examination confirmed absence of malignant or dysplastic features, to ensure that the tissues were fully benign. Stromal cells were isolated as previously described [17]. Briefly, each tissue was minced with scalpel blades and digested in medium containing type I collagenase (0.5 mg/ml) for 12–18 hr at 37°C. The crude suspension of cells was further dissociated by repeated pipetting and then centrifuged at 150*g* for 20 sec to sediment undissociated tissue and clumps of epithelial cells. The supernatant, consisting predominantly of a single-cell suspension of stromal cells, was centrifuged at 200*g* for 1 min, and the supernatant was replaced with medium which comprised RPMI-1640 supplemented with 2 mM L-glutamine, 10% FCS, 100 units/ml penicillin, 0.1 mg/ml streptomycin (Gibco, Life Technologies, Paisley, Scotland, UK), and 0.05 μg/ml each of testosterone and hydrocortisone (Sigma Chemical Co., Poole, Dorset, UK). The cells were seeded onto 9-cm-diameter plastic culture dishes and grown in a humidified atmosphere of 5% CO_2 in air at 37°C with changes of medium three times each week. The cells were serially passaged between 3–5 times before being used in the experiments. In all, 10 cultures derived from different hyperplastic prostate glands were isolated.

Addition of Noradrenaline and Variable Concentrations of Doxazosin

To study the effects of different doses of doxazosin upon cytoskeletal proteins, two cell cultures (designated PR7 and PR9) were selected because previous studies had shown that these stromal cells responded to noradrenaline with a pronounced increase in immunoreactivity to actin and myosin filaments when plated sparsely [17]. The cells from both cultures were released from the plates with trypsin and EDTA, and counted using a hemocytometer, and each was seeded into 24 dishes at a density of 1.5×10^3 cells/cm^2. The cultures were established in similar medium, except that testosterone and hydrocortisone were omitted. The cells were allowed to adhere overnight. The dishes containing each cell line were divided into two groups of 12 each. Group A was destined to receive doxazosin only, whereas plates of group B received doxazosin together with noradrenaline.

Doxazosin mesylate (5.5 mg) (Cardura®, Pfizer, Ltd., Sandwich, Kent, UK) was dissolved in 10 ml sterile distilled water to give a stock 1 mM solution. From this stock, two further solutions of concentrations 10 μM and 0.1 μM were prepared. These solutions were dispensed with a micropipette into 10 ml of medium in each dish to provide a range of five different final concentrations extending from 0.001–10 μM in 10-fold increments. Each increment was added to a pair of dishes from each group. The final pair received no doxazosin. The dishes from group A received no further treatment, but 5 mM noradrenaline bitartrate (Arterenol, Sigma Chemical Co.) were additionally pipetted to all 12 dishes in group B to a final concentration of 20 μM. Both the medium and drugs were replaced on alternate days for a total of 10 days.

In order to more closely approximate the state of stromal cells in the intact prostate, the above procedure was repeated on confluent, nonproliferating cultures of the same two cell lines. To achieve this, cells from both cultures were each seeded onto 24 tissue culture dishes at a density of approximately 6×10^3 cells/cm^2. These were fed with the above medium, but without hormone supplements, until they just reached confluence, after which the medium was replaced with 10 ml of RPMI-1640 containing 2 mM L-glutamine, the above antibiotics, and 1% (v/v) dialyzed and carbon-stripped FCS. Both doxazosin and noradrenaline were added, using an identical regime to that described above.

Harvesting Cells

Cells from all 96 dishes were digested with trypsin/EDTA for 5 min, and cell clumps were disaggregated by repeated pipetting until a monodisperse suspension of cells, confirmed microscopically, was obtained. Trypsin was inactivated by addition of 1 ml FCS. The cells from each plate were washed with phosphate-buffered saline (PBS) and centrifuged before those cells derived from each treatment were pooled and fixed in 2 ml of 70% chilled methanol. Samples of the cell suspension from each type of culture were counted using a hemocytometer.

Addition of Noradrenaline and Fixed Concentrations of Doxazosin

To determine the influence of doxazosin upon immunoexpression of smooth muscle actin and myosin, stromal cells derived from all 10 prostates were examined using identical procedures. Doxazosin was employed at a concentration of 1 μM in preference to the maximum dose of 10 μM, because the previous experiments described above showed that this level reduced the influence of noradrenaline but without inhibiting cell growth.

Cells from each culture were seeded into five petri dishes, grown in medium containing 10% FCS until just confluent, and thereafter fed with medium containing 1% carbon-stripped serum, as described. One dish from each culture acted as control and received no drugs; to a second was added 1 μM doxazosin; to a third, 20 μM noradrenaline; and to a fourth, both doxazosin and noradrenaline. The cells in the final dish were reserved for labelling with isotype-matched antibodies of irrelevant specificity. After 10 days, cells from all of the plates were harvested by trypsin-EDTA digestion and fixed in 1 ml of 70% methanol.

Flow Cytometry

Cells from lines PR7 and PR9, which had received various concentrations of doxazosin, were each aliquoted into five microcentrifuge tubes to provide approximately 0.5×10^6 cells per tube. Cells from the 10 cultures which had received fixed concentrations of doxazosin were each divided into two tubes. In both instances the cells were rehydrated with PBS for 30 min and their supernatants replaced with 200 μl of blocking buffer, comprising 1% bovine serum albumin in PBS, and left overnight at 4°C. Supernatants were replaced with 100 μl of monoclonal antibodies to α-smooth muscle actin, smooth muscle myosin, desmin, talin, and vimentin (Table I). The tubes were incubated at 37°C for 40 min, with occasional agitation to disperse the cells. The antibodies were then withdrawn, and the cells were washed in 1 ml of PBS for 10 min followed by the addition of 100 μl of sheep anti-(mouse IgG)-FITC conjugate diluted 1:250 in blocking

TABLE 1. Nature of Antibodies Used to Label Stromal Cells, Their Isotypes, and Dilutions

Antibody	Isotype	Hybridoma clone	Dilution	Target protein
Alpha-smooth muscle actin	IgG2a	1A4	1:800	Actin myofilaments in smooth muscle cells and myofibroblasts
Smooth muscle myosin	IgG_1	hSM-V	1:200	Contractile myosin filaments in smooth muscle
Desmin	IgG_1	DE-U-10	1:20	Intermediate filament of all muscle cells
Talin	IgG_1	8d4	1:200	Attachment plaque protein of fibroblasts
Vimentin	IgM	VIM 13.2	1:200	10-nm intermediate filaments of all mesenchymal cells

buffer. Incubation occurred for a further 30 min at 37°C, after which supernatants were replaced with PBS. Cells derived from fixed concentrations of doxazosin were also treated as above, except that only antisera to α-smooth muscle actin and myosin were used. Negative control cells, derived from the same cultures, were treated with an antibody of irrelevant specificity but the same isotype as the primary antibody, followed by the fluorescent secondary antiserum.

The fluorescence intensity of each sample of labelled cells was measured using a Becton Dickinson FACSort flow cytometer (San Jose, CA) and analyzed with Lysis software (Becton Dickinson). Sensitivity of the instrument was adjusted so that the mean intensity of the negative controls fell within the first log decade with, at most, 5% lying above this range. A total of 20,000 events was counted, with electronic noise and subcellular debris excluded by setting a threshold on forward light scatter (cell volume). Data were recorded as frequency distribution histograms and mean fluorescence intensity units. Data were analyzed statistically with paired *t*-tests.

Cell-Growth Assay

To determine whether doxazosin and noradrenaline influenced the growth of prostatic stromal cells, cells from cultures PR7 and PR9 were each seeded into four 24-well plates (Nunclon, Roskilde, Denmark) at a density of 5×10^3 cells per cm^2. For the first 48 hr following seeding, the cells were incubated in medium supplemented with 10% FCS to allow firm adherence by the cells. Thereafter, following washing with PBS, each of the wells in two of the plates received 2 ml of RPMI-1640 medium containing 1% dialyzed and carbon-stripped FCS. To the other two plates was added 2 ml of RPMI-1640 medium containing 10% whole FCS. To the wells of all four plates of both cell lines, doxazosin was added in 10-fold increments of concentration (0–10 μM) so that all four wells of a column received the same quantity of drug. Two of these plates containing cells from each culture also received noradrenaline to a standard final concentration of 20 μM. Media were changed twice during 1 week. To perform the measurements at the end of the assay period, media were withdrawn and 0.5 ml of RPMI-1640 containing 1 mg/ml of MTT (Sigma Chemical Co.) were added to each well and incubated at 37°C for 4 hr [18]. The developed blue formazan chromophore was dissolved in 400 μl of dimethylsulphoxide, and aliquots of 195 μl were transferred to a 96-well plate to measure optical absorbance with a Multiskan MS plate reader (Life Sciences International, Basingskoke, UK). Optical absorbance was converted into cell number from a previously-constructed calibration curve.

RESULTS

Effects of Doxazosin on Cell Lines PR7 and PR9

Effects of growth medium on cytoskeletal proteins. In the absence of noradrenaline or doxazosin (basal control conditions), flow cytometric frequency distribution histograms of fluorescence were displaced to the right for cells grown in medium containing 1% stripped FCS when contrasted with the same cells grown in 10% FCS. This shift was associated with increased mean fluorescence intensities of 192% for α-smooth muscle actin, 145% for myosin, 19% for desmin, and 17% for talin, but only 1% for vimentin. When 20 μM noradrenaline were added to cells grown in 1% stripped serum, the mean fluorescence of actin and myosin was further increased to 315% and 175%, respectively, of control cells grown in 10% serum.

The different growth conditions profoundly affected the immunofluorescence frequency-distribution profiles of cells labelled for smooth muscle actin. For both PR7 and PR9 grown in 10% serum, the resulting histogram was asymmetrical with a long right-hand tail (Fig. 1a), indicating that a small proportion of cells exhibited a high expression for actin. When the same cells were grown in 1% stripped serum, the distribution was broad and symmetrical, with a wide apex and numerous cells with high actin

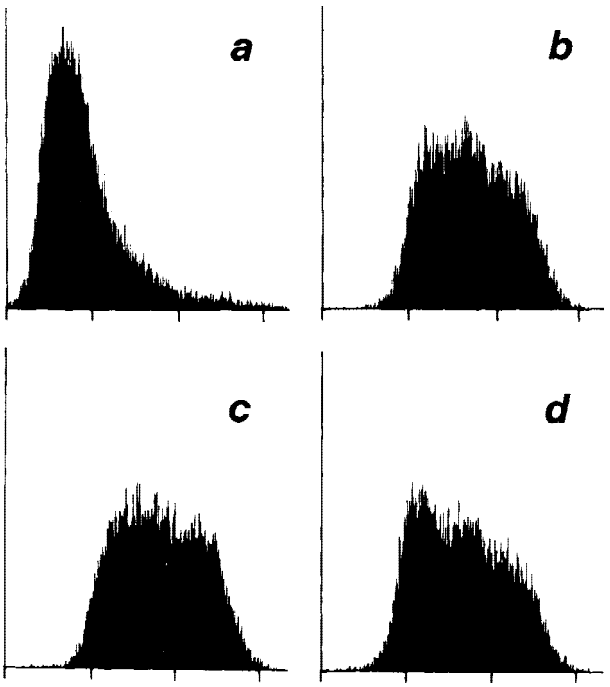

Fig. 1. Flow-cytometric frequency-distribution profiles of PR7 stromal cells labelled for smooth muscle actin. **a:** Untreated control cells grown in medium containing 10% FCS. **b:** Untreated control cells grown in 1% stripped FCS. **c:** Cells grown in 1% stripped serum together with 20 μM noradrenaline. **d:** Cells grown in 1% stripped serum together with 20 μM noradrenaline and 1.0 μM doxazosin. In each histogram, fluorescence intensity units are represented on the logarithmic abscissa, which includes three log decades, and the number of cells are on the ordinate, which spans 110 events.

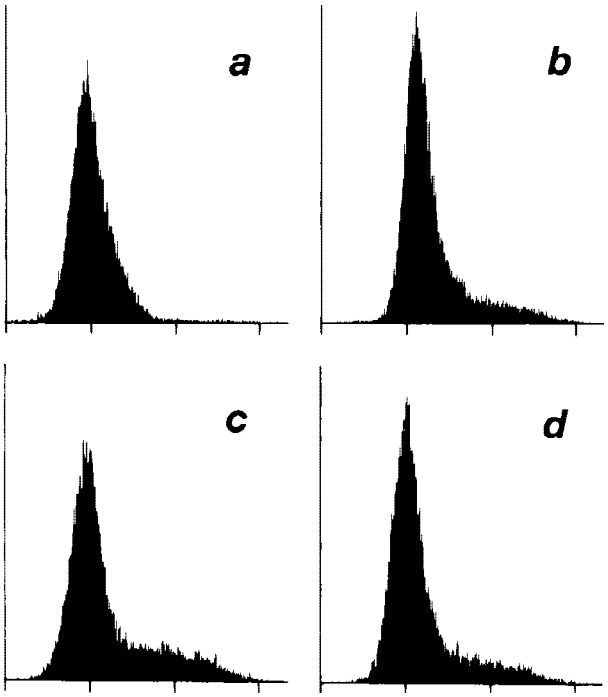

Fig. 2. Flow-cytometric frequency-distribution profiles of PR7 stromal cells labelled for smooth muscle myosin. **a:** Untreated control cells grown in medium containing 10% FCS. **b:** Untreated control cells grown in 1% stripped FCS. **c:** Cells grown in 1% stripped serum together with 20 μM noradrenaline. **d:** Cells grown in 1% stripped serum together with 20 μM noradrenaline and 1.0 μM doxazosin. In each histogram, fluorescence intensity units are represented on the logarithmic abscissa, which includes three log decades, and the number of cells are on the ordinate, which spans 190 events.

expression (Fig. 1b). Myosin labelling of cells grown in 10% FCS showed a narrow peak followed by a fine ''tail'' of high immunofluorescence extending to the right (Fig. 2a). In 1% stripped serum, this tail was accentuated (Fig. 2b). Thus, prostatic stromal cells grown to confluence and rendered quiescent by removal of serum growth factors showed a greatly increased expression of cytoskeletal proteins, especially actin and myosin, when compared with the same but actively-growing cells stimulated by 10% FCS.

Effects of noradrenaline on cytoskeletal proteins. Addition of noradrenaline increased the immunofluorescence intensities of smooth muscle actin and myosin filaments by approximately 30% above that of untreated controls (data not shown). Fluorescence intensities of desmin, talin, and vimentin were generally increased to a lesser amount, and in some cases slightly reduced below control values. When all five cytoskeletal proteins were considered together, noradrenaline induced a mean increase in fluorescence of 21.9% for cells grown in 10% unstripped FCS, but only 12.5% for those cells grown in 1% stripped serum. In neither instance was this increase statistically significant ($P > 0.05$).

Effects of different doses of doxazosin. When fluorescence intensities of all five cytoskeletal filaments were analyzed collectively, cells receiving only doxazosin (group A) showed no significant difference from the untreated controls at any of the concentrations of doxazosin, irrespective of whether the cells were grown in 10% serum or in 1% stripped serum. However, when the differences between cells receiving only noradrenaline and those receiving noradrenaline plus doxazosin (group B) grown in 1% stripped serum were analyzed, the latter were significantly lower ($P < 0.05$) at all concentrations except 1.0 μM. For cells grown in 10% unstripped fetal calf serum, statistical significance was reached only at a concentration of 1.0 μM ($P < 0.05$).

The flow-cytometric frequency-distribution profiles

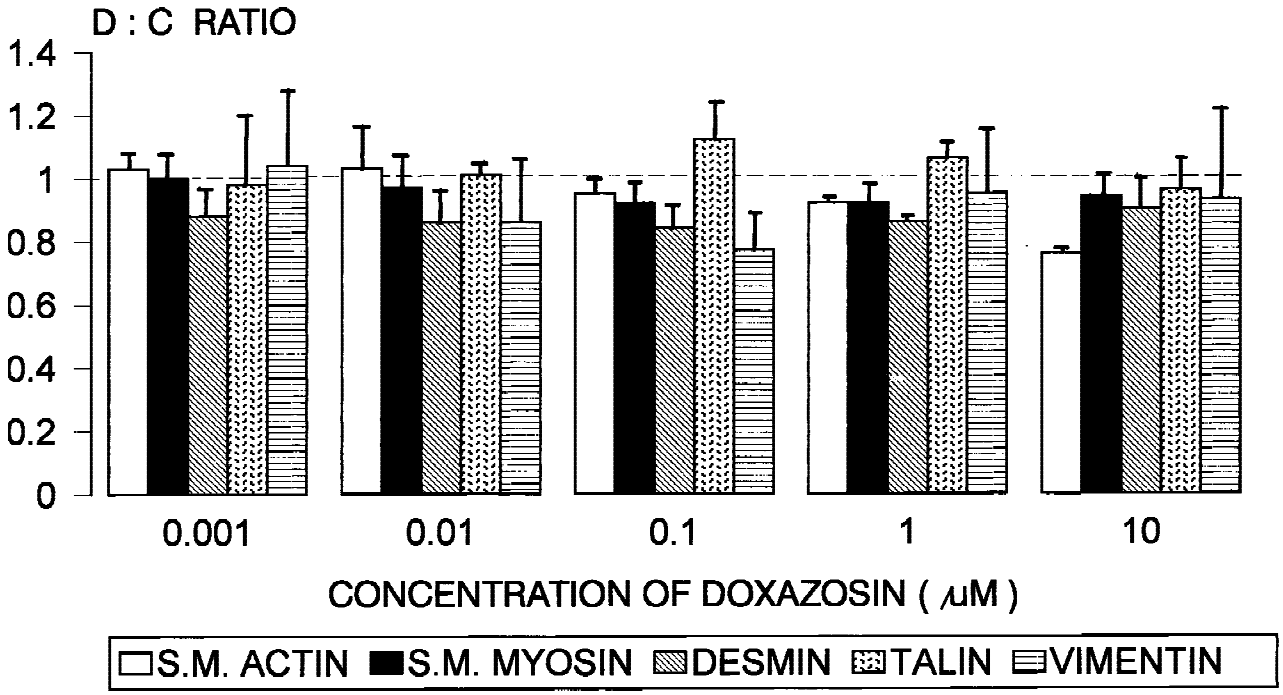

Fig. 3. Block diagram showing the ratio of mean fluorescence intensity of cells treated with doxazosin to that of control cells with no treatment (D:C ratio) for cells grown in medium containing 10% FCS. Here and in Figures 4–6, immunofluorescences of five different cytoskeletal proteins are represented for each of five concentrations of doxazosin. Each column represents the mean value from PR7 and PR9, and error bars define the standard error of the mean. The dashed line indicates a D:C ratio at which no change in immunofluorescence is expected.

for desmin, talin, and vimentin were little altered by addition of noradrenaline or doxazosin, but were simply displaced along the abscissa. However, for smooth muscle actin, addition of noradrenaline caused the appearance of a second peak (Fig. 1c). Addition of noradrenaline plus doxazosin to these cultures resulted in a diminution of this peak so that it became similar to, or lower than, that of the corresponding untreated control (Fig. 1d). Changes in the shape of fluorescence histograms for cells labelled for myosin were less pronounced, with noradrenaline causing an increased proportion of cells included within the right-hand tail (Fig. 2c). Addition of doxazosin caused this tail to diminish so that the distribution resembled that of the control cells (Fig. 2d).

To facilitate comparison between the five cytoskeletal proteins, the fluorescence of cells receiving only doxazosin (D) was expressed as the ratio with respect to that of the fluorescence of untreated control (C) cells for each antibody (D:C ratio). The fluorescence intensities of cells receiving both noradrenaline and doxazosin (ND) were expressed as a ratio with respect to those receiving noradrenaline (N) only (ND:N ratio). These ratios are illustrated as bar charts in Figures 3–6. The effects of different doses of doxazosin upon immunoexpression of five cytoskeletal proteins (D:C ratio) from cells grown in 10% FCS are shown in Figure 3. Many of the ratios were less than the ''zero change'' value of 1.0, although standard error bars of several of them approached or exceeded unity. Addition of both doxazosin and noradrenaline to the medium induced an increase in the ND:N ratios of all filaments, except desmin at 0.001 μM and 0.01 μM doxazosin (Fig. 4). At the three higher concentrations the mean ND:N ratios were generally less than 1.0, with error bars exceeding unity in five instances. Apart from this small difference between low and high concentrations, there was no dose-related response to doxazosin of any of the cytoskeletal filaments.

For stromal cells grown in 1% stripped serum, the D:C ratios were similar to those for cells grown in 10% serum with some values in excess of, and other less than 1.0 (Fig. 5). At 1 μM doxazosin, D:C ratios of desmin and talin were depressed, but actin and myosin showed little change at any concentration. In contrast, the mean ND:N ratios were reduced to a greater extent than those of the D:C ratios (Fig. 6). Taking the error bars into account, 15 of the 25 measurements were less than unity. The greatest difference was at the maximum dose of 10 μM, where immunofluorescences of actin, myosin, and vimentin were markedly reduced, with ND:N ratios of 0.57, 0.70, and 0.59, respectively. However, there was no overall dose-related trend.

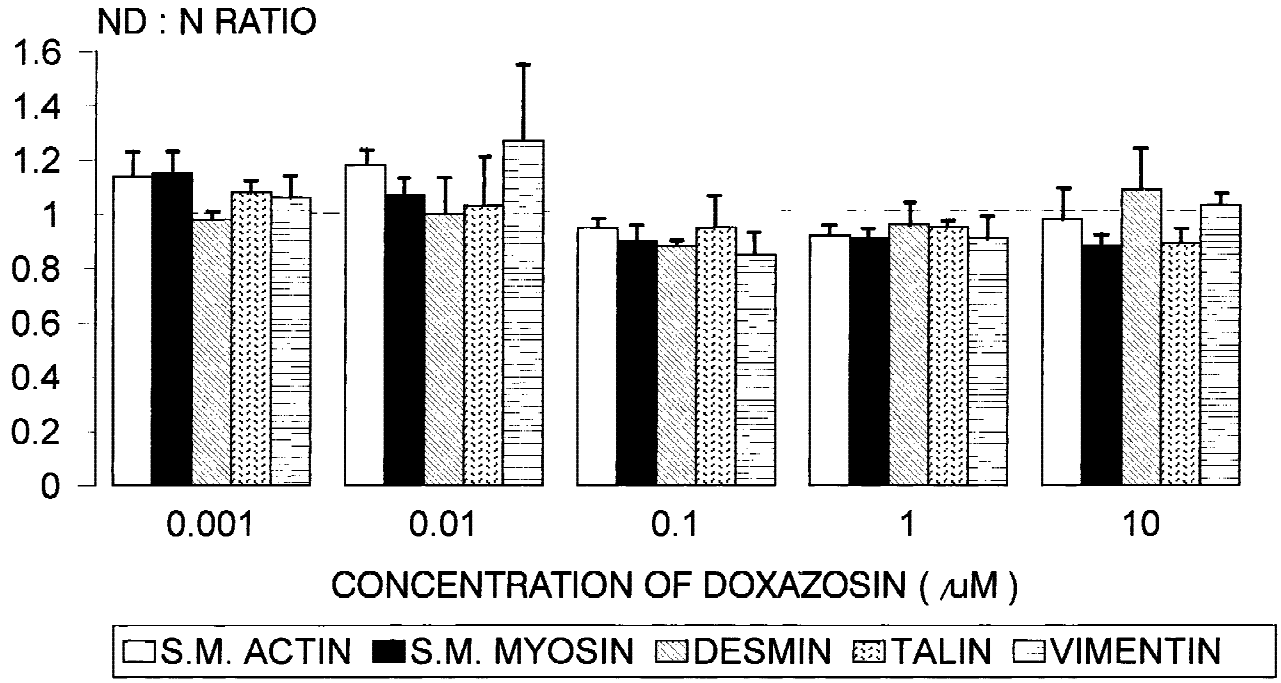

Fig. 4. Block diagram showing the ratio of mean fluorescence intensity of cells treated with doxazosin plus noradrenaline to that of cells with noradrenaline alone (ND:N ratio) for cells grown in medium containing 10% FCS.

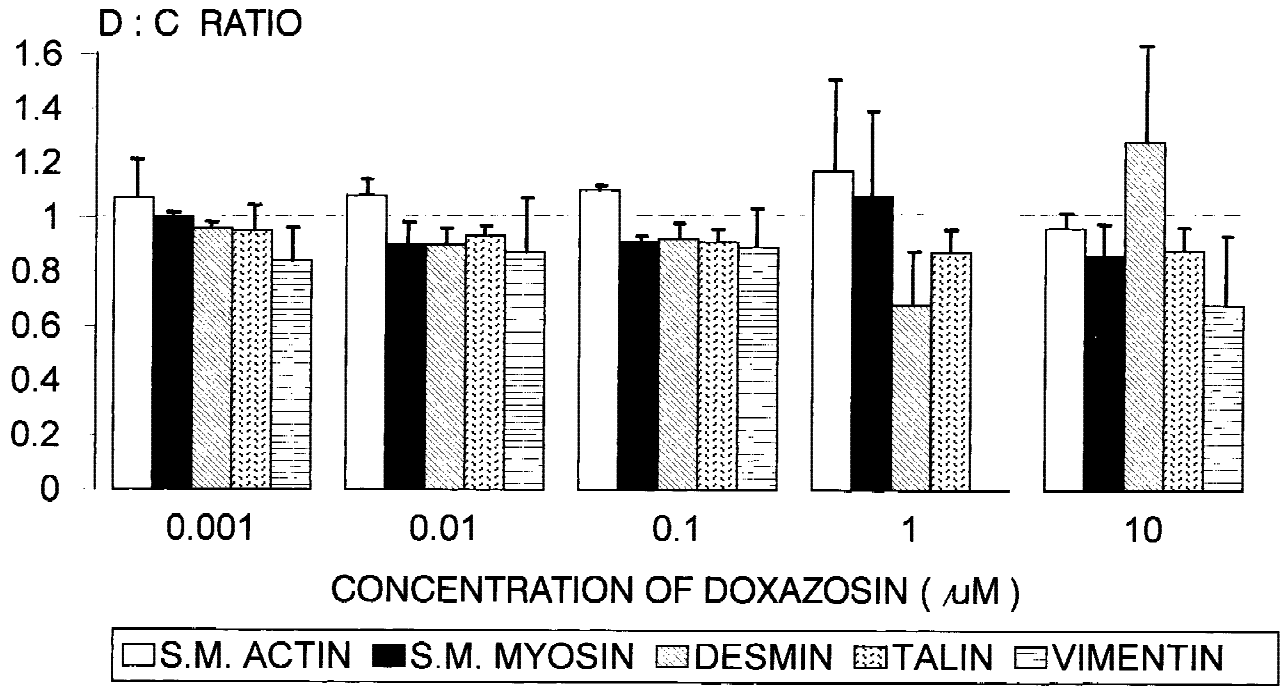

Fig. 5. Block diagram showing the ratio of mean fluorescence intensity of cells treated with doxazosin to that of control cells (D:C ratio) for cells grown in medium containing 1% stripped FCS.

Effects of Fixed Concentration of Doxazosin on 10 Cell Lines

To statistically analyze the effects of doxazosin upon contractile filament protein expression, the immunofluorescence intensities of smooth muscle actin and myosin were analyzed flow-cytometrically in 10 cultures of different prostatic stromal cells. The results of these experiments are presented in Table II. Doxazosin induced a small reduction in fluorescence of

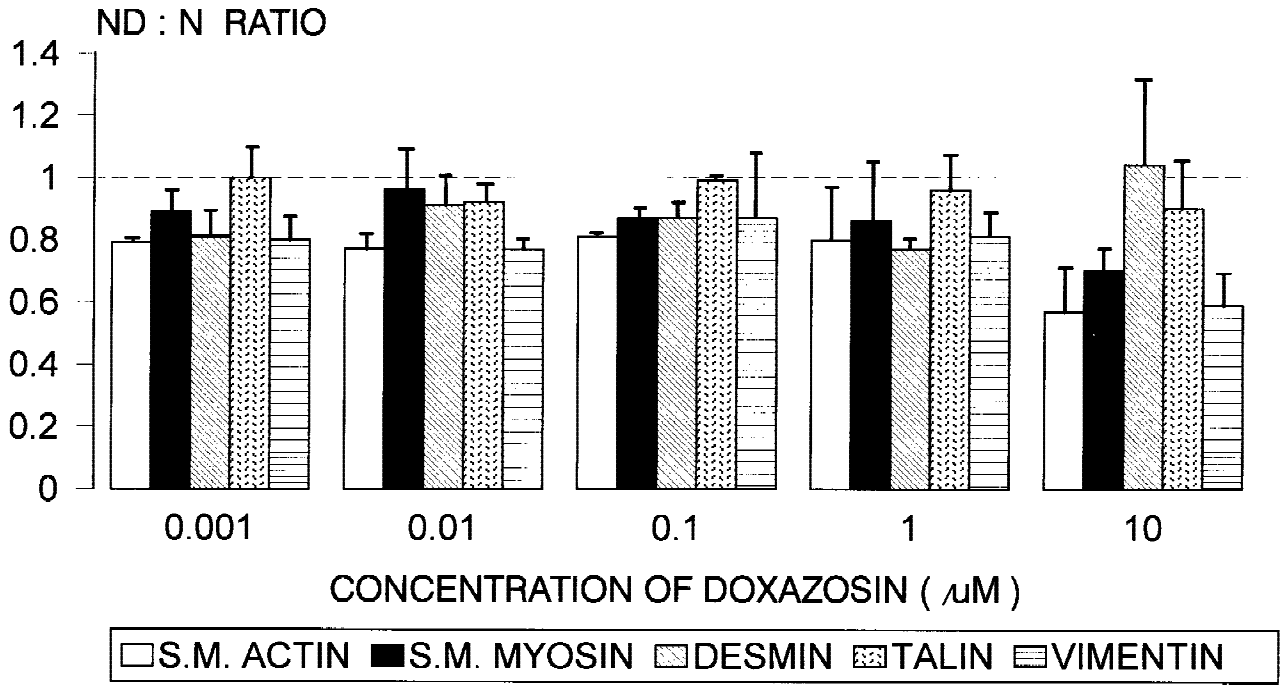

Fig. 6. Block diagram showing the ratio of mean fluorescence intensity of cells treated with doxazosin plus noradrenaline to that of cells with noradrenaline alone (ND:N ratio) for cells grown in medium containing 1% stripped FCS.

TABLE II. Mean Fluorescences and Ratios of 10 Stromal Cell Lines Labelled for Smooth Muscle Actin and Myosin

Cell line	Control	Doxazosin	Noradrenaline	Doxazosin + noradrenaline	$\frac{D}{C}$	$\frac{N}{C}$	$\frac{ND}{C}$
Smooth muscle actin							
PR1	136.3	138.2	155.8	102.3	1.01	1.14	0.75
PR3	110.4	81.1	91.6	98.6	0.73	0.83	0.89
PR7	283.5	177.5	170.8	146.9	0.63	0.60	0.52
PR9	63.0	59.8	117.1	81.8	0.95	1.86	1.30
PR10	186.7	181.5	214.3	187.8	0.97	1.15	1.01
PR11	102.2	108.6	95.7	108.8	1.06	0.94	1.06
PR12	405.2	438.7	496.7	496.2	1.08	1.23	1.22
PR13	75.4	80.8	76.3	74.5	1.07	1.01	0.99
PR18	130.6	129.6	199.3	164.4	0.99	1.53	1.26
PR20	279.1	269.6	436.1	386.3	0.97	1.56	1.38
Smooth muscle myosin							
PR1	18.8	19.4	20.0	17.3	1.03	1.06	0.92
PR3	28.1	29.8	27.2	29.3	1.06	0.97	1.04
PR7	36.3	33.2	36.8	33.6	0.91	1.01	0.93
PR9	35.1	33.1	55.0	47.9	0.94	1.57	1.36
PR10	36.5	43.5	47.6	43.7	1.19	1.30	1.20
PR11	28.9	30.6	25.4	23.6	1.06	0.88	0.82
PR12	94.7	93.8	106.3	107.1	0.99	1.12	1.13
PR13	34.1	35.2	37.9	34.5	1.03	1.11	1.01
PR18	28.4	22.2	43.3	39.2	0.78	1.52	1.38
PR20	85.1	77.7	109.3	72.9	0.91	1.28	0.86

cells labelled for smooth muscle actin when compared with untreated controls in 6 out of the 10 cell lines, but this reduction was not statistically significant. Addition of noradrenaline to the medium resulted in an increase in fluorescence in all but three cell lines (PR3, PR7, and PR11), but this was not significantly different from the controls. Addition of doxazosin plus noradrenaline reduced the fluorescence from that ob-

tained with noradrenaline alone, in eight of the cell lines, a difference which was significant ($P < 0.01$).

Similar changes were found when cells were labelled for smooth muscle myosin, although mean fluorescence intensities were smaller (Table II). While there was no significant difference in the fluorescence intensities of cell lines treated with doxazosin alone, noradrenaline induced a significant ($P < 0.025$) increase above control in all but two of the cell lines. As with actin, doxazosin plus noradrenaline reduced this fluorescence intensity but not significantly. In Table II, fluorescence intensities for each of the treatments are normalized by their expression as ratios with respect to those of the control cells. Noradrenaline alone (N:C) was associated with a variable ratio for both actin and myosin, with some cell lines responding more strongly to noradrenaline than others. This ratio was significantly higher than the ratio of doxazosin-only to control (D:C) for both actin ($P < 0.025$) and myosin ($P < 0.05$). Although the ND:C ratio was in most cases substantially lower than the N:C ratio for both filament proteins, many ratios remained above unity, indicating that the influence of noradrenaline was incompletely inhibited by doxazosin. Nevertheless, the ND:C ratio was statistically significantly lower than the N:C ratio for both actin and myosin ($P < 0.05$ and $P < 0.025$, respectively).

Cell-Growth Assay

When stromal cells were grown in medium containing 10% FCS, doxazosin had no effect upon growth at any of the concentrations used. This lack of antagonist effect was not modified by the presence of noradrenaline. For cells grown in medium plus 1% stripped serum, doxazosin had no effect upon growth at concentrations from 0.001–1.0 μM. However, at the highest concentration (10 μM) there was severe inhibition of growth of both cell lines, with cell numbers falling to between 5–6% of those in the other wells ($P < 0.05$). The presence of 20 μM noradrenaline did not alter this figure with PR7, but for PR9 the number of viable cells in 10 μM doxazosin was increased to 30% of that of the other wells ($P < 0.01$).

DISCUSSION

This investigation confirms our previous findings [17], that exposure of human prostatic stromal cells to noradrenaline in the physiological range induces increased expression of cytoskeletal filament protein. It also confirms the observation that noradrenaline has a greater influence upon these filaments when cells are sparsely distributed and actively growing, in contrast to confluent cultures. In the present study, stromal cells were cultured in conditions which more closely approximate their state in the intact, hyperplastic prostate gland by first allowing their growth to confluence and then restricting further growth by replacement of 10% FCS by 1% carbon-stripped serum in the medium. Such quiescent, unstimulated cells showed a greater than twofold increase in expression of actin and myosin filaments when compared with the same cells growing in 10% FCS. This finding is in accord with the observation that proliferating smooth muscle cells contain mainly synthetic organelles but, once division ceases, they adopt a contractile phenotype [19,20], thus indicating the majority of cells in cultures PR7 and PR9 to be of smooth muscle phenotype.

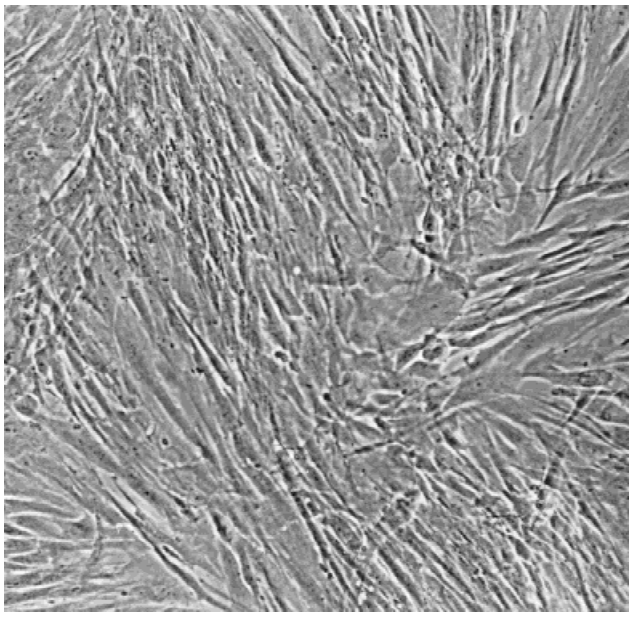

Fig. 7. Confluent culture of prostatic stromal cells from PR11. Most of the cells are narrow and attenuated with a fibroblastic morphology (phase contrast, ×220).

Data from 10 different confluent cultures of prostatic stromal cells grown in 1% stripped serum indicate that doxazosin alone induced an insignificant reduction in expression of actin and myosin filaments. Conversely, noradrenaline produced, on average, 18.5% and 18.4% increases in expression of actin and myosin, respectively, which was significantly different in the latter. However, the response to noradrenaline was variable, with an N:C ratio for actin which ranged from 0.60–1.86. These observations probably reflect the heterogeneous composition of the prostatic stroma between different subjects [3,4]. Such differences were evident on microscopic examination of the cells in culture where, for example, PR11 comprised predominantly attenuated filiform cells of fibroblastic morphology (Fig. 7) which showed a small decrease in actin expression with noradrenaline. Conversely,

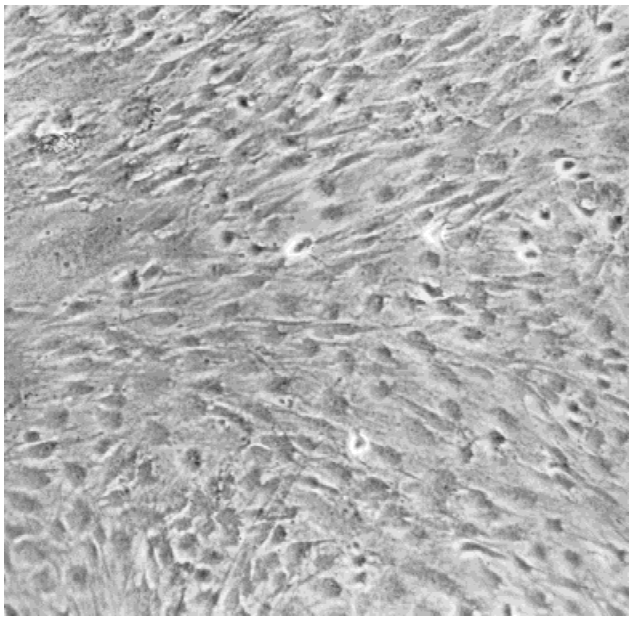

Fig. 8. Confluent culture of prostatic stromal cells from PR20. Cells are elongated but are broader and less tapered than those of PR11. Their morphology is characteristic of smooth muscle (phase contrast, ×220).

PR20 comprised shorter, broader cells of smooth muscle morphology (Fig. 8) in which noradrenaline induced a pronounced rise in expression of actin.

Simultaneous addition of noradrenaline and doxazosin at fixed concentrations to the culture media caused mean reductions in expression of actin and myosin of 10.1% and 9.4%, respectively, from those produced by noradrenaline alone. Thus, blockade of α_1-adrenergic receptors by doxazosin not only inhibits but also reverses the phenotypic modulation which follows exposure to noradrenaline. However, within this system is hysteresis of phenotypic modulation, since the observed reversal is not always complete, as shown by some ND:C ratios greater than unity (Table II). The other three intermediate cytoskeletal filaments (desmin, talin, and vimentin) appeared to be affected to a lesser degree by noradrenaline and doxazosin. Of the three, vimentin showed the greatest reduction in expression in response to doxazosin (Fig. 6).

No consistent dose response to doxazosin was identified for any of the cytoskeletal proteins, despite a 10^4-fold range of concentration used in the experiments. Thus, with 1% stripped serum at the lowest concentration of 0.001 μM doxazosin, expression of all filaments except talin was reduced. Tenfold increments of doxazosin concentration failed to induce any further change until the highest concentration of 10 μM (Fig. 6). At the lowest concentration, it is possible that doxazosin blocked all of the α_1-adrenoceptors on smooth muscle cells so that higher doses were without further effect. Furthermore, periodic replenishment of the medium with fresh agents may have provided more molecules of doxazosin to displace noradrenaline from adrenoceptor sites in addition to those already blocked. The abrupt fall in expression of actin, myosin, and vimentin at 10 μM doxazosin may have been due to toxic side effects of the drug at this concentration, as suggested by the data from the growth assay.

The apparent absence of any dose-related modulation of cytoskeletal proteins by doxazosin is at variance with pharmacological studies on the effect of α_1-adrenergic blocking drugs upon the contractile response of prostatic tissues, hence indicating at least two different mechanisms. Tension studies performed in vitro on prostates of animals and men demonstrated that α_1-adrenoceptor antagonists, such as prazosin or tamsulosin, competitively inhibit contraction induced by noradrenaline or electrical stimulation. This inhibition occurs in a dose-dependent fashion [21–27]. Doxazosin also shows a dose-dependent inhibition of contraction of the human prostate [25]. It may be, therefore, that binding of α_1-adrenoceptor antagonists to stromal smooth muscle cells affects their contraction and expression of myofilaments differently. If the noradrenaline-induced increase in cytoskeletal filaments is due simply to sustained contraction of the cells, doxazosin should oppose this increase in a dose-dependent manner. However, the relationship between concentration of noradrenaline and filament expression is not linear but biphasic, with an initial fall followed by a progressive rise in immunofluorescence [17]. These data suggest that noradrenaline affects stromal cell filaments by a mechanism other than prolonged contraction, and that the influence of doxazosin on cytoskeletal filament expression may not necessarily be related to its concentration.

Another difference between contraction and phenotypic modulation is their time scale, with relaxation of myofilaments occurring rapidly, whereas changes to myofilament density would be expected to occur over a period of several days. In our experiments, stromal cells were in contact with noradrenaline and doxazosin for 10 days so that, when harvested, it is likely that changes to their phenotype were already complete. Any dose-dependent effect which doxazosin may have upon the *rate* of cytoskeletal modulation would not be apparent in our experiments. Nevertheless, we can conclude that, by the time equilibrium is established in this model system, noradrenaline increases the expression, and hence also the number, of contractile filaments within prostatic stromal cells, and that this effect is greatly inhibited by doxazosin.

The conclusions from this study may be of clinical relevance to patients receiving doxazosin to relieve

obstructive BPH, since the range of concentrations of doxazosin used in this study also included the range of likely therapeutic concentrations in patients. Thus, the reported plasma concentrations in a group of patients receiving the maximum recommended dose of 4 mg of doxazosin daily peaked at 42 ng/ml (0.075 μM), with an average of 25 ng/ml (0.045 μM) [28]. Previous work showed that noradrenaline stimulates a greater increase in immunoexpression of cytoskeletal proteins in stromal cells, which are sparsely distributed and actively multiplying compared with confluent cultures [17,29]. The present study indicates that doxazosin has minimal influence upon such active cells; hence, it is unlikely to inhibit noradrenaline-induced increases in cytoskeletal proteins while the stroma continues to proliferate. However, α_1-adrenergic blocking agents are administered to patients when urethral obstruction is clinically manifest, by which time stromal enlargement may be established, compact, and relatively quiescent. Addition of doxazosin to tissue cultures which simulated this situation resulted in a significant reduction in expression of actin and myosin filaments when compared with the effects of noradrenaline alone. This reduced synthesis of contractile elements should diminish the force generated when stromal cells are stimulated by noradrenaline to contract. Thus, one might speculate that the therapeutic use of doxazosin, and other α-adrenergic antagonists, not only inhibits the noradrenergic contraction of prostatic stromal smooth muscle cells but also reduces their capacity to contract in the first instance. This is a hitherto unsuspected benefit of this type of agent in the management of patients with BPH.

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