

# Cyclic stretch induces human bladder smooth muscle cell proliferation *in vitro* through muscarinic receptors

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**Abstract.** The present study aimed to investigate whether the cyclic stretch-induced proliferation of human bladder smooth muscle cells (HBSMCs) is mediated by muscarinic (M) receptors, together with the signal transduction mechanisms involved in this process. HBSMCs seeded onto silicone membranes were subjected to different cyclic stretches (5, 10, 15 and 20%) for 6 and 12 h. As the effect of cyclic stretch on M2 and M3 mRNA expression levels was maximal at 6 h 10% stretch, all subsequent experiments were performed at this stretch. Western blot analysis was used to quantify M2, M3, protein kinase C (PKC) and phosphorylated (p)-PKC protein expression levels, flow cytometry was employed to examine cell cycle distribution and a 5-bromo-2-deoxyuridine (BrdU) incorporation assay was used to assess cell proliferation at this stretch. Subsequently, HBSMCs were exposed to different acetylcholine concentrations and/or cyclic stretch, M receptor antagonists [AF-DX16, an M2 receptor antagonist; 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP), an M3 receptor antagonist and atropine, a non-selective antagonist] and GF 109203X, a PKC antagonist, to assess the possible underlying signaling mechanisms. Cyclic stretch was found to increase the proliferation of HBSMCs and the expression levels of M2, M3, PKC and p-PKC proteins. M receptor and PKC antagonists exerted no apparent effect on nonstretched cells, but reduced the incorporation of BrdU into stretched cells; the most pronounced effects were observed when non-selective M receptor and PKC antagonists were applied. Notably, 4-DAMP did not inhibit stretch-induced PKC activation. These results indicate that the activation of the M3 receptor signaling pathway in stretch-induced HBSMC proliferation occurs via PKC-independent mechanisms.

## Introduction

The primary function of the urinary bladder is to store urine at low intravesical pressures and expel it periodically via a coordinated well-maintained physiological contraction. As a dynamic smooth muscle organ, the urinary bladder is continuously subjected to mechanical stimuli, including hydrodynamic pressure and stretch, which have been shown to be required for the growth and development of the urinary bladder (1). However, a pathological mechanical environment, resulting from bladder outlet obstruction (2), neurological disease (3) and bladder overactivity, may result in a hyperplastic or hypertrophic response in the detrusor smooth muscle and increased extracellular matrix production, followed by a deleterious change in bladder function. A number of studies (4-6) have demonstrated that cyclic stretch induces cell proliferation, and several signaling pathways, including the phosphoinositide 3-kinase (PI3K)/Akt (7), p38 (8), signal transducer and activator of transcription 3 (9) and extracellular signal-regulated kinase (ERK)1/2 (10) signaling pathways, have been investigated as possible molecular mechanisms underlying the cell proliferation induced by cyclic stretch. However, thus far, whether the proliferation of human bladder smooth muscle cells (HBSMCs) resulting from cyclic stretch is mediated by muscarinic (M) receptors has, to the best of our knowledge, not been demonstrated.

A previous study (11) demonstrated that exposure of HBSMCs to sustained hydrostatic pressure may result in increased expression levels of muscarinic (M) receptor M2 and M3 subtypes in a time- and pressure-dependent manner. This result prompted the evaluation of the effect of cyclic stretch on M receptor expression levels in the present study. To investigate whether the proliferative effect occurs via M receptors, experiments were performed at the degree of stretch that yielded maximally increased expression levels of M receptors and this stretch was used in all subsequent experiments. Acetylcholine has been demonstrated to exert a mitogenic effect on numerous cell types, although studies have focused on neuronal (12-14) and tumor cell types (15-17). Studies analyzing the mitogenic effect of acetylcholine on HBSMCs are limited (18). In the present study, the proliferative effect of acetylcholine and/or cyclic stretch on HBSMCs was assessed, along with whether the proliferative effect is mediated by muscarinic signaling pathways. The cell cycle distribution was also specifically quantified, and the effect of M receptor and protein kinase C (PKC)

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antagonists on HBSMC proliferation was evaluated following cell exposure to cyclic stretch, in order to identify the possible underlying mechanisms involved.

The present study, by evaluating the role of the M signaling pathway on HBSMC proliferation induced by cyclic stretch, may enrich understanding of the possible molecular mechanisms of signaling pathways for cell proliferation resulting from cyclic stretch, together with the cellular and molecular changes that affect the bladder wall, and provide novel targets for specific medications or therapies of a number of urinary bladder diseases.

## Materials and methods

**HBSMC culture.** HBSMCs (catalog no. 4310; ScienCell, Carlsbad, CA, USA) were expanded in culture using low-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, HyClone; Thermo Fisher Scientific), penicillin (100 U/ml) and streptomycin (100 µg/ml). These cells were plated in 75-cm<sup>2</sup> culture flasks and cultured at 37°C, in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The medium in the flasks was changed every 48 h and the cells were passaged every 2-3 days. All experiments were performed on cells between passages 3 and 7.

**In vitro cyclic stretch.** The HBSMCs were seeded onto silicone membranes for 24 h prior to transfer to a bioreactor (BioDynamic; Bose Corporation, Framingham, CA, USA) chamber. The treated silicone membrane was subjected to 0.1 Hz cyclic stretch with a 1:1 stretch/relaxation ratio sine wave stretch pattern. A control silicone membrane was placed in the same chamber without stretch. Initially, the effects of cyclic stretch were examined at 5, 10, 15 and 20% stretch for 6 and 12 h, respectively. The effect of cyclic stretch on M2 and M3 mRNA expression levels, which were subsequently determined by reverse transcription polymerase chain reaction (RT-PCR), was maximal at 10% stretch for 6 h. Therefore, all subsequent experiments were performed at this degree of stretch for 6 h, to investigate whether cyclic stretch causes proliferation of HBSMCs via M receptors.

**RNA isolation and RT-PCR.** Total RNA was isolated from control and treated HBSMCs using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted RNA was dissolved in nuclease-free water and then quantified by measuring the absorbance at 260 nm using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA was then synthesized using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. M2 and M3 mRNA expression levels were quantified by RT-PCR analysis with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. RT-PCR was performed using SYBR Premix EX Taq premix reagent [Takara Biotechnology (Dalian) Co., Ltd., Dalian, China] and a Bio-Rad iQ5 detection system (Bio-Rad). The reactions were performed under the following conditions: 94°C for 3 min, 40 cycles at 94°C for 5 sec, 54°C for 30 sec and 72°C for 20 sec. PCR product quality was monitored using post-PCR melt curve analysis. The sequences of primers used in RT-PCR were as follows: GAPDH sense, GCTTCGCTCTCT-

GCTCCT; GAPDH antisense, CGCCCAATACGACCAAAT; M2 sense, AGCAAACATGCATCAGAATTGG; M2 antisense, GTGCACAAAAGGTGTTAATGAG; M3 sense, ACCCAGCTCCGAGCAGATGGAC; and M3 antisense, CGGCTGACTCTAGCTGGATGG.

**Flow cytometric analysis of the cell cycle profile.** Once the stretch procedure was complete, the control and treated HBSMCs were harvested. The cells were washed in cold phosphate-buffered saline (PBS) twice and then fixed in 70% ethanol overnight at 4°C. Following centrifugation at 25°C and 241 x g for 3 min, the cells were washed with cold PBS, and gently resuspended in 500 µl PBS containing 100 µg/ml RNaseA and 50 µg/ml propidium iodide for 30 min in the dark. The cells were then diluted with PBS and flow cytometry was performed using a Cytomics FC500 flow cytometer (Beckman Coulter, Miami, FL, USA). All samples were assayed in triplicate and the cell apoptotic rate was calculated as follows: Apoptotic rate (%) = (apoptotic cell number/total cell number) x 100. In addition, cell proliferation was calculated as follows: Proliferation index (%) = (S+G2/M)/(G0/G1+S+G2/M) x 100.

**5-Bromo-2-deoxyuridine (BrdU) incorporation assay.** HBSMCs from each group were harvested and then suspended at a concentration of 4x10<sup>5</sup> cells/ml with DMEM. Cell suspensions were transferred to 96-well plates (200 µl/well), 10 µM 5-bromo-2-deoxyuridine labeling solution (BrdU Cell Proliferation ELISA kit; Roche Applied Science, Penzberg, Germany) was added to each well and the incubation was continued for 16 h. The cells were fixed and exposed to anti-BrdU (1:100) antibody and substrate solutions. Proliferation was quantified by measuring the absorbance value at 450 nm wavelength using an ELISA plate reader (Model 680; Bio-Rad). For the acetylcholine experiments, various concentrations of acetylcholine (10 nM-100 µM) were added following harvesting of the HBSMCs from each group. For the M and PKC receptor antagonist experiments, 1 µM of the M antagonists 4-DAMP (sc-200167) for the M3 receptor antagonist, AF-DX116 (sc-223772) for the M2 receptor antagonist and atropine for the non-selective antagonist (All from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and 5 µM PKC antagonist (GF 109203X; sc-24003; Santa Cruz Biotechnology, Inc.) were added for 1 h prior to the HBSMCs being subjected to cyclic stretch.

**Western blot analysis.** The M2, M3, PKC and phosphorylated (p)-PKC protein expression levels in HBSMCs were detected by western blot analysis using GAPDH as an internal standard. For antigen retrieval, total cellular protein was extracted from control and treated cells using cell lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 100 µg/ml phenylmethanesulfonylfluoride and 1% Triton X-100. Following removal of cell debris by centrifugation at 12,000 x g for 5 min, 50 µg of each lysate sample was boiled for 5 min in sample buffer, separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Pall Corporation, Port Washington, NY, USA). Nonspecific reactivity was blocked by incubating the membrane in 5% non-fat dry milk in TBST containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20 for 1 h at room temperature. The membrane was then incubated with specific primary

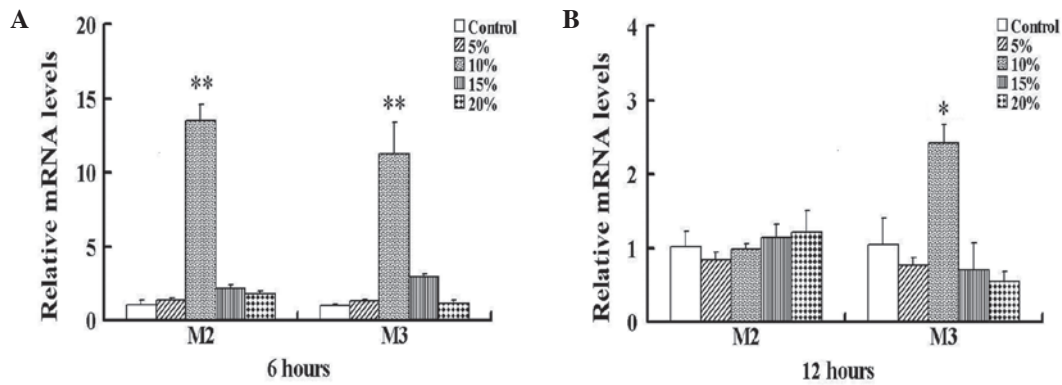


Figure 1. Comparisons of M2 and M3 muscarinic receptor mRNA expression levels between nonstretched and stretched human bladder smooth muscle cells following exposure of the cells to (A) 6 h and (B) 12 h cyclic stretch. Glyceraldehyde-3-phosphate dehydrogenase served as the internal standard. Values indicate mean  $\pm$  standard deviation for each condition from three independent experiments. \*\* $P < 0.01$  vs. 6 h control, \* $P < 0.05$  vs. 12 h control.

antibodies at 4°C overnight followed by secondary anti-rabbit IgG (Jackson ImmunoResearch Inc., West Grove, PA, USA) for 1 h. Reactive protein was detected using an enhanced chemiluminescence system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The antibodies used for western blotting were anti-GAPDH, polyclonal, rabbit anti-human M2 (ab123421), polyclonal mouse anti-human M3 (ab167566) and polyclonal rabbit anti-human PKC (ab69531) antibodies and polyclonal rabbit anti-human p-PKC antibodies (ab195769). All antibodies were at a dilution of 1:1,000 and were purchased from Abcam (Cambridge, MA, USA).

**Statistical analysis.** In the BrdU incorporation assays, triplicate culture wells were used and each experiment was repeated at least three times. All statistical tests were conducted using SPSS software, version 11.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance tests and paired t-test were used to determine significant differences ( $P < 0.05$ ) between experimental samples and controls. Data are expressed as the mean  $\pm$  standard deviation.

## Results

**Expression levels of M receptor subtypes following cyclic stretch in vitro.** The exposure of HBSMCs to 6 h cyclic stretch *in vitro* resulted in significantly increased expression levels of M2 and M3 mRNA at 10% stretch, compared with those of the control nonstretched HBSMCs ( $P < 0.01$ ; Fig. 1A). No significant differences between nonstretched and stretched cells were identified below or over 10% stretch. Following exposure to 12 h cyclic stretch, M2 mRNA expression in the HBSMCs declined to the initial levels; however the M3 mRNA expression levels remained significantly increased compared with those of the control cells ( $P < 0.05$ ; Fig. 1B). As the effect of cyclic stretch on the expression levels of M2 and M3 mRNA was maximal at 6 h 10% stretch, all subsequent experiments were performed at this stretch. Western blot analysis also demonstrated a marked increase in M2 and M3 receptor protein expression levels in response to this stretch (Fig. 2).

**Effect of cyclic stretch on cell cycle and cell proliferation.** Compared with the control group, the numbers of S and

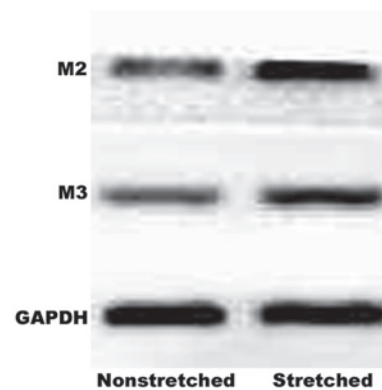


Figure 2. Representative autoradiography images of western blots revealing M2 and M3 muscarinic receptor protein expression levels in nonstretched and stretched human bladder smooth muscle cells (HBSMCs). The cells underwent 10% cyclic stretch, or negative control treatment for 6 h. Compared with the nonstretched HBSMCs, the expression levels of M2 and M3 proteins were significantly increased in the stretched HBSMCs. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal standard.

G2/M phase HBSMCs were increased in the cyclic stretch group, as shown by representative samples of the flow cytometric data in Fig. 3A and B. HBSMC apoptosis was inhibited. The rate of apoptosis was reduced from  $16.8 \pm 0.42\%$  in the control to  $4.1 \pm 0.22\%$  following cyclic stretch ( $P < 0.01$ ; Fig. 3C), while the cell proliferation index was increased from  $27.6 \pm 0.76\%$  in the control to  $71.5 \pm 0.31\%$  following cyclic stretch ( $P < 0.01$ ; Fig. 3D). In conclusion, the data suggest that the exposure of HBSMCs to cyclic stretch inhibits apoptosis and stimulates proliferation.

**Effect of acetylcholine and/or cyclic stretch on BrdU incorporation in HBSMCs.** Exposure of HBSMCs to different concentrations of acetylcholine, ranging between 10 nM and 100  $\mu$ M, resulted in increased cell proliferation, as measured by BrdU incorporation, compared with the control in a concentration-dependent manner, reaching significance at 100  $\mu$ M ( $P < 0.05$ ; Fig. 4A). In addition, when 10 or 100  $\mu$ M acetylcholine was administered to stretched HBSMCs, statistically significant increases in BrdU incorporation were detected compared with nonstretched HBSMCs treated with the same respective acetylcholine doses ( $P < 0.05$ ; Fig. 4B). Exposure of

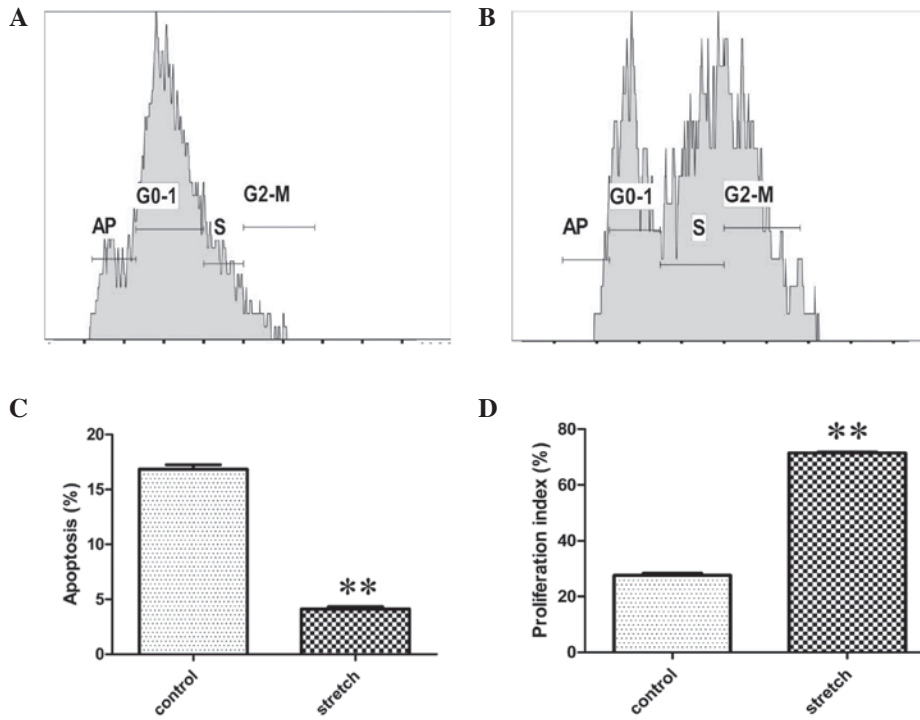


Figure 3. Representative control and treated sample flow cytometric data. Human bladder smooth muscle cells were subjected to 10% cyclic stretch, or negative control treatment for 6 h. Compared with (A) control sample cells, (B) the numbers of treated cells in the S and G2/M phases were increased following cyclic stretch. Cyclic stretch also induced (C) a significant reduction in the apoptotic rate and (D) a significant increase in the proliferation index. Data are expressed as mean  $\pm$  standard deviation. \*\* $P < 0.01$  vs. control.

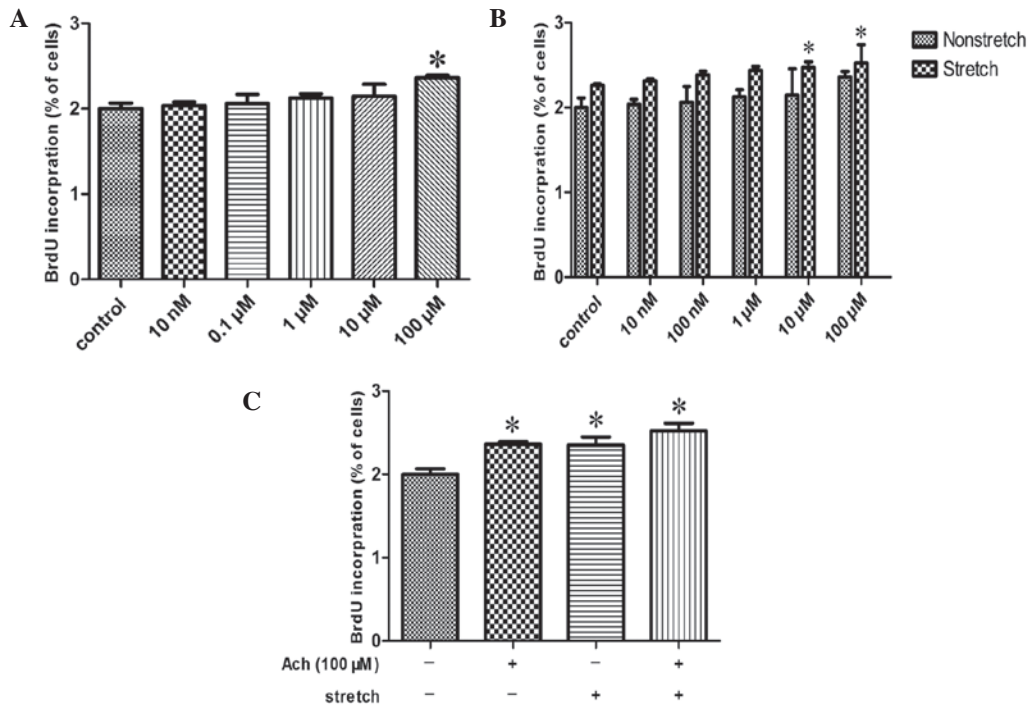


Figure 4. 5-Bromo-2-deoxyuridine (BrdU) incorporation into human bladder smooth muscle cells (HBSMCs) at various concentrations of acetylcholine (10 nm-100  $\mu$ M) and/or 6 h 10% cyclic stretch. (A) BrdU incorporation into nonstretched HBSMCs following exposure to different concentrations of acetylcholine. (B) BrdU incorporation into stretched and unstretched HBSMCs at various acetylcholine concentrations. (C) BrdU incorporation following exposure to 100  $\mu$ M acetylcholine and/or cyclic stretch. \* $P < 0.05$  vs. control.

HBSMCs to 100  $\mu$ M acetylcholine and/or cyclic stretch induced significant increases in cell proliferation compared with the control ( $P < 0.05$ ; Fig. 4C). BrdU incorporation following

exposure to 100  $\mu$ M acetylcholine, cyclic stretch, or 100  $\mu$ M acetylcholine and cyclic stretch was increased by 17.4, 17.6 and 26.1%, respectively.

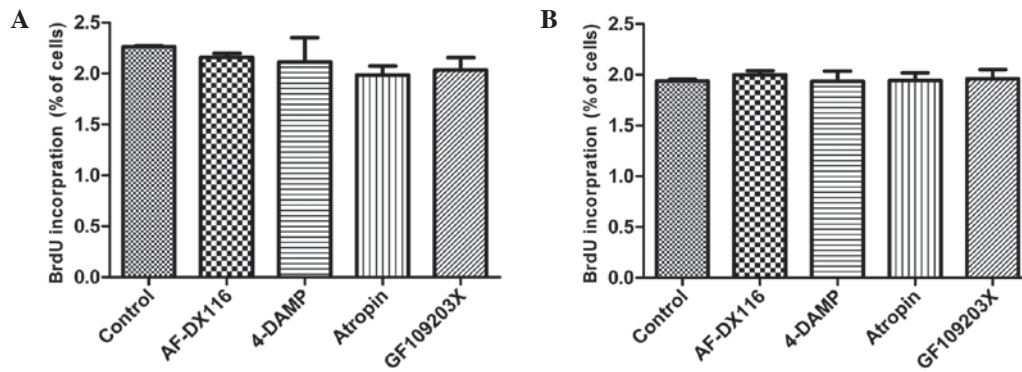


Figure 5. Effect of muscarinic and protein kinase C (PKC) antagonists on 5-bromo-2-deoxyuridine (BrdU) incorporation into (A) stretched and (B) non-stretched human bladder smooth muscle cells.  $P > 0.05$  vs. control. 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide.

*Effect of muscarinic and PKC antagonists on HBSMCs.* In order to investigate whether M receptor signaling pathways are involved in a possible mechanosensitive mechanism for the cell proliferation induced by cyclic stretch, 1  $\mu$ M M receptor antagonist [AF-DX16, M2 receptor antagonist; 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP), M3 receptor antagonist; and atropine, non-selective antagonist) was added to compare the effects of M receptor antagonists on stretched and nonstretched HBSMCs. Stretched cells exhibited a reduction in BrdU incorporation when exposed to M antagonists, although these reductions failed to reach statistical significance (Fig. 5A). By contrast, M receptor antagonists exerted no clear effect on nonstretched HBSMCs (Fig. 5B).

As the results of the experiments involving M2 and M3 receptor antagonists suggest that the cell proliferation induced by cyclic stretch is caused primarily by activation of the M3 receptor, the effect of PKC, a predominant downstream effector of M3 receptor signaling pathways, on the proliferation of HBSMCs was analyzed. To determine the involvement of PKC, 5  $\mu$ M PKC antagonist (GF 109203X) was administered to nonstretched and stretched HBSMCs. As shown in Fig. 5A, BrdU incorporation following the exposure of stretched HBSMCs to GF 109203X was reduced compared with that in the control cells, although this reduction failed to reach statistical significance. Nonstretched cells exhibited no change in BrdU incorporation in the presence of GF 109203X (Fig. 5B). Following exposure to AF-DX16, 4-DAMP, atropine and GF 109203X, BrdU incorporation was reduced by 8.4, 10.2, 15.8 and 13.6%, respectively, in stretched cells.

*Involvement of stretch-activated PKC in the M3 receptor signaling pathway.* PKC is an important downstream effector of the M3 receptor signaling pathway and p-PKC is the activated form. PKC is physiologically activated by diacylglycerol (DAG) in a process that occurs subsequent to activation of the prototypical M3 receptor signaling pathway. In bladder smooth muscle, this may contribute to contraction and proliferation. The BrdU assay indicated that GF 109203X reduces stretch-induced HBSMC proliferation. In order to investigate whether activation of PKC via the M3 receptor signaling pathway is involved in the proliferative effect of cyclic stretch, western blot analysis was used to quantify PKC and p-PKC expression levels in nonstretched and stretched HBSMCs. During stretching, the cells were treated in the presence

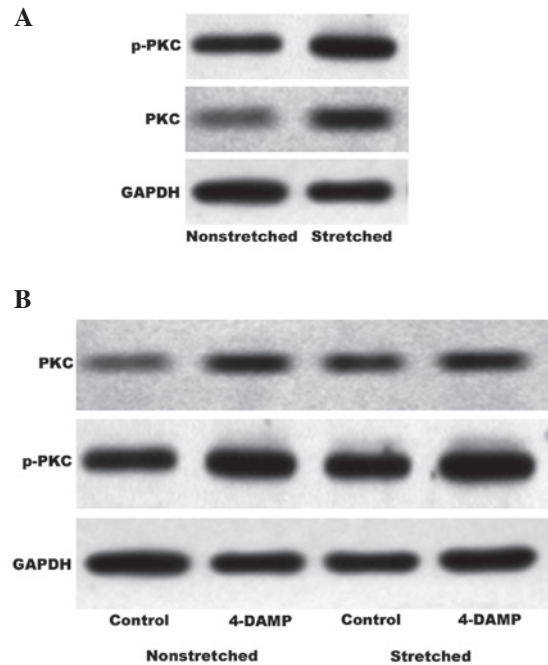


Figure 6. Representative autoradiography of western blots for protein kinase C (PKC) and phospho (p)-PKC expression levels in nonstretched and stretched human bladder smooth muscle cells (HBSMCs). During stretching, the cells were treated in the presence or absence of 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide 4-DAMP. (A) Compared with the nonstretched HBSMCs, PKC and p-PKC expression levels were clearly increased in the stretched HBSMCs. (B) Notably, the observed increases in PKC and p-PKC expression levels induced by stretching were not inhibited by 4-DAMP. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal standard.

or absence of 4-DAMP. As shown in Fig. 6A, cyclic stretch enhanced the expression and activation of PKC. Notably, the observed increases in PKC and p-PKC expression levels induced by stretching were not inhibited by 4-DAMP (Fig. 6B).

## Discussion

Abnormal function of the urinary bladder, resulting from outlet obstruction or neurogenic bladder, may result in high intravesical pressure and constant abnormal stretch of the bladder wall. The constant abnormal stretch, marked by profound molecular and cellular level changes, may alter the mechanical

and functional properties of the bladder (19). To achieve an improved understanding of the underlying cellular and molecular mechanisms, the role of the muscarinic signaling pathway on HBSMC proliferation induced by cyclic stretch was investigated. In order to find out the most effective cyclic stretch parameters, the impact of cyclic stretch on M2 and M3 mRNA expression levels was examined at 0, 5, 10, 15 and 20% stretch for 6 and 12 h, respectively. Previous studies have reported that M2 and M3 receptor density may be upregulated or downregulated in response to an abnormal mechanical environment (unstable bladder) generated by outlet obstruction or neurogenic bladder in different *in vivo* and *ex vivo* models (20-22). However, these studies simply examined M2 and M3 receptor density at a single degree of stretch. The present study revealed dynamic changes in M2 and M3 mRNA expression levels in response to different cyclic stretches, and that this expression was upregulated to the maximum extent at 10% stretch for 6 h. These results support the hypothesis that the expression levels of M2 and M3 receptor subtypes may signify a dynamic process in the development of an unstable bladder, which also may be a possible reason for the differences in the results of previous reports.

Proliferation and apoptosis are considered to be opposing cellular processes that mediate the response of the bladder to short-term obstructive stimuli (23). The present study, using HBSMCs *in vitro*, demonstrated that mechanical deformation regulates these two cellular processes. A significant increase in the proliferation of HBSMCs was accompanied by a significant reduction in the rate of apoptosis in response to cyclic stretch. These findings are consistent with those of Galvin *et al* (4), who found that mechanical stretch at 12.3% significantly reduced HBSMC apoptosis *in vitro* as early as 6 h after an apoptotic peak at 3 h, which was continued following a 48-h stretch. The authors suggested that, in contrast to the low rates of apoptosis *in vivo*, the high basal rate of spontaneous apoptosis of HBSMCs *in vitro* produced opposite results from those of *in vivo* models. Similar results were obtained by Estrada *et al* (24) in an *ex vivo* model bladder smooth muscle cell system, indicating that stretch may be an antiapoptotic stimulus in bladder smooth muscle cells.

Acetylcholine has been extensively investigated with regard to its role as the primary neurotransmitter responsible for bladder contraction. In addition, a number of studies have demonstrated the mitogenic effect of acetylcholine on numerous cell types (25-27). However, to the best of our knowledge, no study thus far has demonstrated the mitogenic effect of acetylcholine on stretched HBSMCs *in vitro*. The results from the present study show that acetylcholine exerted a mitogenic effect on HBSMCs in a concentration-dependent manner. Following 10 or 100  $\mu\text{M}$  acetylcholine treatment, the cells that were exposed to cyclic stretch exhibited statistically significant increases in cell proliferation compared with nonstretched HBSMCs at these acetylcholine concentrations. Lee *et al* (18) observed similar results following 1  $\mu\text{M}$  acetylcholine treatment. The possible reasons for this discrepancy include different experimental methods and conditions, and different time periods of exposure to acetylcholine. The finding that acetylcholine and stretching exerted a greater mitogenic effect than stretch treatment alone suggests that cyclic stretch induces a mitogenic acetylcholinergic effect on

HBSMCs. Possible reasons for this effect include the upregulation of M2 and M3 receptor expression and an increased number of activated M receptor targets. Thus, the proliferative effect on HBSMCs of acetylcholine and/or cyclic stretch may be through the activation of M receptors.

With regard to the effect of M antagonists on BrdU incorporation in stretched and nonstretched HBSMCs, the stretched cells exposed to M receptor antagonists demonstrated a reduction in BrdU incorporation compared with a negative control, while nonstretched cells exhibited no evident changes among these groups. These results support the hypothesis that the proliferative effect on stretched cells was due to the activation of M receptors. As the relative BrdU incorporation activity was reduced to the greatest extent by 4-DAMP and atropine, stretch-induced HBSMC proliferation was considered to be caused primarily by activation of the M3 receptor, although the contribution of the M2 receptor cannot be ruled out. PKC is a major downstream signaling kinase of the prototypical M3 receptor signaling pathway. BrdU incorporation in the presence of PKC antagonist was reduced; furthermore, western blotting results revealed that cyclic stretch significantly upregulated PKC and p-PKC expression, demonstrating the possible involvement of PKC in stretch-stimulated cell proliferation. However, PKC activation was shown, through western blot analysis, to occur in response to stretch in the presence of 4-DAMP, indicating that PKC activation following stretching is independent of the M3 receptor signaling pathways. This finding is notable for two reasons. The apparent independence of the M3 receptor signaling pathway in stretch-induced PKC activation appears inconsistent with the prototypical M3 receptor signaling pathway. This finding also contrasts with a previous study, which observed that PKC activation via the M3 receptor signaling pathway induced cell proliferation in other types of cells (12). Another study suggested that stimulation of the M3 receptors activates adenylate cyclase, phospholipase A2, inositol triphosphate and DAG. Each of these molecules in turn activates different signaling pathways (28). The PI3K and ERK intracellular signaling pathways have also been demonstrated to regulate, at least partially, M receptor-mediated cell proliferation (29). Combined with the results of the present study, this suggests that cyclic stretch induced HBSMC proliferation via the M3 receptor signaling pathway through the activation of downstream signaling pathways other than the PKC signaling pathway. In conclusion, these findings indicate that stretch-stimulated HBSMC proliferation occurred via multiple independent signaling pathways and that the M3 receptor signaling pathway was involved in this process by PKC-independent mechanisms.

In conclusion, cyclic stretch was demonstrated to induce HBSMC proliferation mediated by the M receptor. Furthermore, the signal transduction mechanism for this process primarily involves the M3 receptor signaling pathway in a PKC-independent manner. These data suggest that the M receptor is more deeply involved in the pathological processes of bladder outlet obstruction and neurogenic bladder than has been previously considered. Therefore, M receptor antagonists should not only be considered for treating the stretch-injured bladder but also for stopping or reversing the cellular changes that affect the bladder wall. In addition, a highly selective M3 antagonist may be more effective than a M2 antagonist. As the PKC antagonist reduced stretch-induced HBSMC proliferation,

its molecular target may be of significance in the treatment of such patients. The potential downstream signaling pathways of M3 receptor activation involved in stretch-induced cell proliferation require identification in further studies.

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