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The purinergic component of human bladder smooth muscle cells' proliferation and contraction under physiological stretch

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ABSTRACT

Objective: To investigate whether cyclic stretch induces proliferation and contraction of human smooth muscle cells (HBSMCs), mediated by P2X purinoceptor 1 and 2 and the signal transduction mechanisms of this process.

Methods: HBSMCs were seeded on silicone membrane and stretched under varying parameters; (equibiaxial elongation: 2.5%, 5%, 10%, 15%, 20%, 25%), (Frequency: 0.05 Hz, 0.1 Hz, 0.2 Hz, 0.5 Hz, 1 Hz). 5-Bromo-2-deoxyuridine assay was employed for proliferative studies. Contractility of the cells was determined using collagen gel contraction assay. After optimal physiological stretch was established; P2X1 and P2X2 were analyzed by real time polymerase chain reaction and Western Blot. Specificity of purinoceptors was maintained by employing specific inhibitors; (NF023 for P2X1, and A317491for P2X2), in some experiments.

Results: Optimum proliferation and contractility were observed at 5% and 10% equibiaxial stretching respectively, applied at a frequency of 0.1 Hz; At 5% stretch, proliferation increased from 0.837 ± 0.026 (control) to $1.462 \pm 0.023\%$, $p < 0.05$. Mean contraction at 10% stretching increased from $31.7 \pm 2.3\%$, (control) to $78.28 \pm 1.45\%$, $p < 0.05$. Expression of P2X1 and P2X2 was upregulated after application of stretch. Inhibition had effects on proliferation (1.232 ± 0.051, $p < 0.05$ NF023) and (1.302 ± 0.021, $p < 0.05$ A314791) while contractility was markedly reduced (68.24 ± 2.31, $p < 0.05$ NF023) and (73.2 ± 2.87, $p < 0.05$ A314791). These findings shows that mechanical stretch can promote magnitude-dependent proliferative and contractile modulation of HBSMCs in vitro, and P2X1 and 2 are at least partially responsible in this process.

1. Introduction

The human bladder is a dynamic organ both in its structure and function. A strong body of data has emerged implicating mechanical stimuli as key regulators of cellular structure and functioning in normal and diseased states [1]. These mechanical stimuli, including strain, pressure, and shear stress can regulate bladder smooth muscle cells (BSMC) phenotype, functionality, and matrix remodeling [2] and even cell survival [3]. During voiding cycles, the bladder wall is predisposed to range of mechanical stretches [4] for which the BSMC must develop homeostatic mechanisms in order to regulate its biological activities.

Similarly, in the urodynamically overloaded bladder; mechanical stretch stress is considered the trigger inducing responses such as bladder hypertrophy and detrusor over activity as result of outlet obstruction [5]. Thus with its involvement in both physiologic and pathologic conditions, it's imperative to explore the mechanisms with which BSMCs perceive external mechanical stimulation (i.e., mechanotransduction) [6–8].

In vitro stretch models have been used to mimic the urodynamically overloaded bladder. Drawing inspiration from such previous studies but altering the design in (a) investigate a more wide and systematic range of stretches and frequencies encompassing all the values previously reported in literature. (b) Use human BSMCs as compared with other studies which were carried out on animal subjects [9,10] to account for heterogeneity between species. (c) More closer resemblance with bladder cycle of continence and micturition because BSMCs undergo a more static type of stretch as the bladder fills up [11]; as compared with previous experiments which were carried out by applying cyclic (sinusoidal or square) pattern of stretch (observed in vascular SMCs). Secondly, under obstructive load, the stretch will slowly hike up and continue for hours as compared with vascular SMCs for which it lasts a mere few seconds. (d) Cyclic filling and emptying ensures normal development of bladder. Thus our model ensures a more physiologic recreation of bladder in vitro.

Adenosine triphosphate (ATP) and acetylcholine (Ach) act on purinergic and muscarinic receptors to initiate detrusor contraction [12], ATP being the primary nonadrenergic, noncholinergic

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(NANC) neurotransmitter [13]. In human detrusor muscle samples lacking any contractile pathology, nerve mediated contractions were significantly abolished by atropine implying that Ach is the primary activating neurotransmitter. Interestingly in samples which displayed pathological contractile over activity, atropine-resistance emerges, with the residual contraction blocked by α,β-methylene ATP, an analogue of ATP [14–16] Thus giving rise to an interesting question; that what role purinergic signaling plays in proliferative and contractile mechanisms in normal HBSMCs where none were reported previously to as much as 40% in pathologic conditions such as interstitial cystitis, outflow obstruction and most types of neurogenic bladder [17–20].

ATP is co-released with Ach from parasympathetic nerves and in the bladder activates purinergic receptors for which it is the primary and most potent ligand; to initiate contraction. ATP binds to purinergic, P2 receptors which in turn are divided into P2X and P2Yfamilies [21]. Ionotropic P2X receptors have 3 peptide units surrounding an ion-permeable pore, admitting Ca^{2+}, Na^{+} and K^{+} entry into the cell upon ATP binding. Although the mRNA of all seven known subtypes (P2X1–7) exist within bladder, P2X1 is most abundant [22].

P2X1 is associated with detrusor smooth muscle membranes and there is intracellular P2X2 immuno-reactivity in detrusor muscle [23].

The interaction of ATP and its receptors is recognized as a crucial component of lower urinary tract sensory and motor control. We will proceed from creating physiologic stretch pattern, establishing optimum proliferation and contraction to deducing the role which purinoceptors play in these important cellular functions of normal BSMCs which are amplified several times in the pathologic bladder.

2. Materials and methods

2.1. Materials

The HBSMCs were ScienCell, USA, Cat. No.4310). Cyclic strain was applied using BOSE Bioreactor (BOSE, BioDynamic, USA). The BrdU cell proliferation assay kit was purchased from Roche (Basel, Switzerland). Contractile function of cells was determined by Cell Contraction Assay (Collagen-based) (Cell Biolabs Inc, California, USA) and Carbamoylcholine chloride (Sigma–Aldrich, USA). RT-PCR reagents were: RNAiso Plus reagent (TaKaRa, Dalian, China), SuperScript II(TaKaRa, Dalian, China), SYBR Premix EX Taq premix reagent (TaKaRa, Dalian, China). Anti-P2X1, P2X2 antibodies were all purchased from Abcam Inc, Cambridge, MA). The P2X1, P2X2 inhibitors; NF023 and A314791 respectively, were from Santa Cruz, California, USA.

2.2. Methods

2.2.1. Cell culture and cellular characterization

HBSMCs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 95% air/5% CO_2. All experiments were performed on cells between passages 3 and 7.

2.2.2. Application of physiological stretch

HBSMCs were seeded onto a silicone membrane. Cells achieving 90% confluence were subjected to stretch with the computer-controlled mechanical stretch unit (BOSE, BioDynamic, USA). Under the control of customized software, the computer operated stretch system was able to recreate bladder filling by applying elongation from 0% to 2.5% during first 3 h, followed by a progressive increase up to 5%, 10%, 15%, 20% or 25% during the last hour(completing a 4 h cycle) according to experiment designed. The bladder emptying was then mimicked by decreasing very quickly from 5%, 10%, 15%, 20% or 25% to 0%, in a few seconds. This stretch wave, articulated in three parts, being cyclically repeated 4 times in 24 h, whereas the cells remained at rest for the remaining time (8 h, in order to take into account the normal lower bladder activity during the night. HBSMCs in the control group were maintained in the same chamber without any stretch. Real-time read-outs from the control software were recorded versus time.

2.2.3. BrdU incorporation experiment for proliferation

After application of cyclic strain, cells were harvested and transferred at a density of 4.0×10^5 per well into 96-well plates. BrdU was added at a concentration of 10 μM to label the cells statically for another 3 h. After centrifugation ($300g \times 10$ min), cells were fixed with FixDenat solution for 30 min at room temperature, and incubated with anti-BrdU peroxidase working solution (freshly diluted 1:100) for 90 min. Following 3 rinses with washing buffer, 100 μL/well substrate solution was added onto the cells and incubated at room temperature. Absorbance at 490 nm was measured at 5 min and every 10 min thereafter in an ELISA plate reader (Bio-Tek uQuant, USA). The optimal value was obtained at 30 min.

2.2.4. RNA isolation and real-time PCR

Real-time fluorescence quantitative PCR (RT-PCR) was used to measure P2X1, P2X2 mRNA in Bio-Rad iCycler (Bio-Rad, Hercules, CA, USA). Total RNA was isolated from cells after strain exposure using an RNAiso Plus reagent. RNA was eluted in 20 μL of nuclease-free water and stored at −70 °C before use. cDNA was synthesized using the Super Script II according to the manufacturer's protocol. Reverse transcription and amplification was performed using the SYBR Premix EX Taq premix reagent, housekeeping gene GAPDH was applied as an internal control. The sequences of the primers used for RT-PCR were as follows:

GAPDH, forward, 5′-GCTTCGCTCTCTGCTCCT-3′; reverse, 5′-CGCCCAATACGACCAAAT-3′; P2X1, forward, 5′-CCCACCATGGCACGG-CGGTTCCAGGAGG-3′ reverse TCAGGATGTCCTCATGTTCTCCT-GCAGG; P2X2, forward 5′-CACAGACGGGTACCTGAAGC-3′, reverse 5′ CGACGGAAGTCAGAGCTGTG-3′.

PCR cycling conditions were 94 °C for 3 min and 40 cycles (94 °C for 5 s, 54 °C for 30 s and 72 °C for 20 s). Data analysis was performed by a comparative cycle threshold method in Bio-Rad iQ5 software.

2.2.5. Measurement of cell contractility by collagen gel contraction assay

To determine gel contraction, a standard kit assay was used [24]. Briefly, 2 parts of cells (2×10^5/mL) were mixed with 8 parts of collagen gel lattice mixture and plated in 24-well plates for 1 h at 37 °C. After the gel was polymerized, 1 mL of medium was added and incubated for 2 days. Carbamoylcholine chloride (1×10^{-4}M) was added, and after 1 h, stressed matrices were released from the surrounding brim of the wells. Areas of collagen gel size were analyzed with ImageJ software and normalized to areas of the well. The degree of contraction was evaluated by determining the surface area of the gel matrix before and after treatment. Data were expressed as a percentage of the uncontracted gel size.

2.2.6. Western blotting

Total cellular protein was extracted and pooled by using Nonidet P-40 (NP-40) protein isolation solution (0.5% NP-40, 60 mmol/L KCl, 1 mmol/L DTT, 10 mmol/L PMSF, 10 mmol/L Tris, pH 8.0, 1 mmol/L ethylene diaminetetraacetic acid, pH 8.0, and 1 mmol/L leupeptin, pepstatin and aprotinin). Protein concentration was calculated using ELISA Read. Membranes were probed with the following antibodies: P2X1, P2X2, GAPDH (all antibodies were 1:1000 diluted) After 2 h incubation with alkaline

phosphatase conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) at room temperature, the signal was detected by the enhanced chemiluminescence method. Exposures were conducted on BioMax MR-1 film (Kodak, New York, NY, USA).

2.2.7. Statistical analysis

All experiments were performed in triplicates. Means ± SD were calculated. One-way ANOVA was applied to compare results between two groups. $p < 0.05$ was considered statistically significant. Figure data are expressed as the mean, the error bars indicates standard deviation.

3. Results

3.1. Stretch induces proliferation in magnitude and frequency dependent manner

In BrdU assay, the absorbance values directly correspond to amount of DNA synthesis and thus the proliferating cells. As depicted in Fig. 1, proliferation activity was enhanced in each group compared with control (elongation = 0, 0.837 ± 0.026). The highest proliferation attained was 1.462 ± 0.023 at 5% stretch and 0.1 Hz frequency. From figure, one can establish that 10%, 15%, 20%, 25% stretch group had considerably up-regulated proliferation (1.312 ± 0.035%; 1.223 ± 0.028%; 1.916 ± 0.065%; 1.017 ± 1.019%; respectively) ($p < 0.05$), although proliferation values experienced a slow decline from 5% stretch group onwards to 25% stretch group. All subsequent experiments were performed at this optimum stretch, i.e. 5% elongation and 0.1 Hz frequency.

3.2. Minor role of P2X1 and P2X2 in cyclic stretch induced proliferation

After establishing optimum physiologic stretch, the P2X1, P2X2 inhibitors, NF023 and A314791 respectively; were applied. Cells were incubated with 10 μM NF023 and 10 μM A314791; 2 h before application of stretch in fresh medium and without addition of fetal bovine serum. Cells from stretch and control group were then assessed with BrdU assay (Fig. 1B). Mean proliferation compared with control group (1.462 ± 0.023) decreased to (1.232 ± 0.051, $p < 0.05$ NF023) and (1.302 ± 0.021, $p < 0.05$ A314791).

These results augment our other research carried in our lab, (Cyclic stretch induces proliferation of human bladder smooth muscle cells by muscarinic receptors in vitro, Yi dai et al. article in press) implying a dual role of purinergic and muscarinic receptors on proliferation).

3.3. P2X1 and P2X2 are involved in contractile modulation of bladder smooth muscle cells

To assess P2X1 and P2X2 influence on contractility of BSMC, cells incubated with 10 μM NF023 and 10 μM A314791 were stretched at 10% elongation and 0.1 Hz for 24 h and compared with control group (10% elongation and 0.1 Hz, no inhibitor added); thirty minutes after releasing collagen gel. Mean contractility decreased from (78.28 ± 1.45%, control group) to (68.24 ± 2.31%, $p < 0.05$ NF023) and (73.2 ± 2.87, $p < 0.05$ A314791) as seen in Fig. 2. The data suggests a strong influence of the predominant P2X1 receptor which is also associated with detrusor motor response as compared with P2X2; being largely implicated in sensory functions.

3.4. Cyclic stretch induces increased P2X1 and P2X2 expression

Up-regulated expressions were observed for P2X1and P2X2. P2X1 (1.47 ± 0.56-fold, $p < 0.05$) and P2X2 (3.61 ± 0.43-fold, $p < 0.05$), mRNA expression compared with non-stretch group as shown in Fig. 3(A). Expressions of mRNA were verified with western blotting. In response to cyclic stretch, protein levels for P2X1 (0.870 ± 0.145, control) and P2X2 (0.8714 ± 0.148, control) increased (1.56 ± 0.245, $p < 0.05$) and (1.9145 ± 0.241, $p < 0.05$) respectively as shown in Fig. 3(B and C). P2X1 inhibitor nullified the stretch effect (0.86 ± 0.112, $p > 0.05$), while P2X2 was still up regulated (0.241 ± 1.14, $p < 0.05$). These data suggested P2X1 and 2 are implicated in stretch-induced proliferation and contraction of HBSMCs.

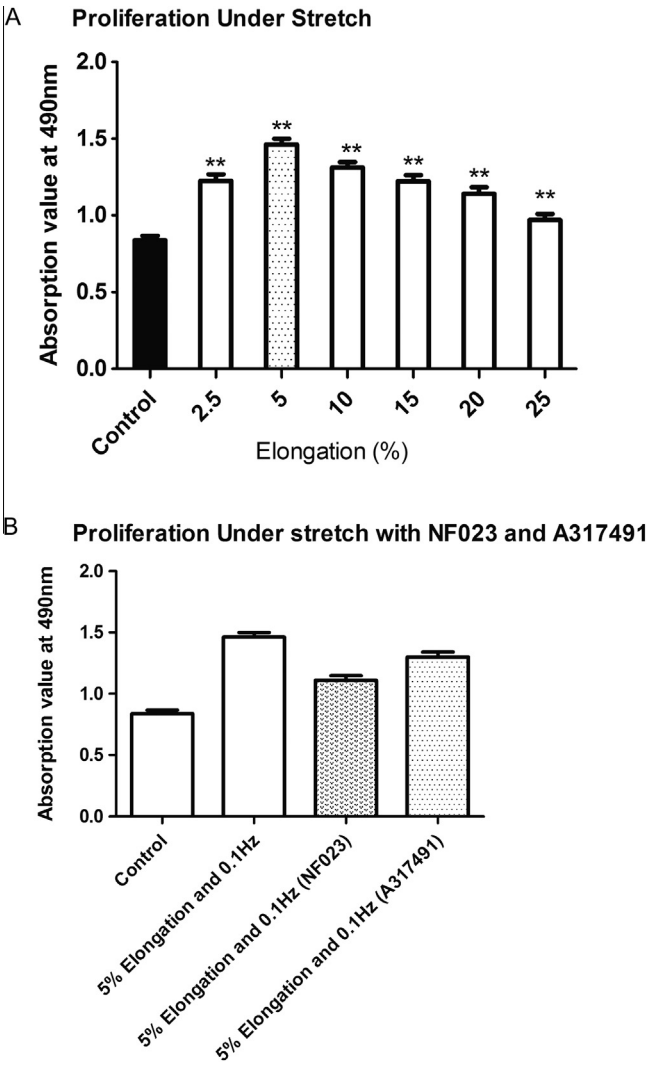

Fig. 1. Mean (+SD) proliferation of bladder smooth muscle cells. Figure shows absorbance values of 5-bromo-2-deoxyuridine (BrdU) incorporation into the newly synthesized DNA and thus the proliferating cells. (A) Represent the absorbance values of different elongations applied at 0.1 Hz, with maximum proliferation observed at 5% elongation, while all other stretches induced considerable influence on proliferation. After application of stretch: with respective inhibitors of P2X1 (NF023) and P2X2 (A317491) the maximum proliferation is reduced as shown in B. Implying a small role for P2X1 and P2X2 in bladder smooth muscle cells.

4. Discussion

We tried to establish a physiologic stretch model which will mimic the conditions under which normal bladder operates in its filling-voiding cycles. The range of elongations and frequencies applied; encompass a broad range of values as reported in literature before us. Optimum proliferation and contractility profiles

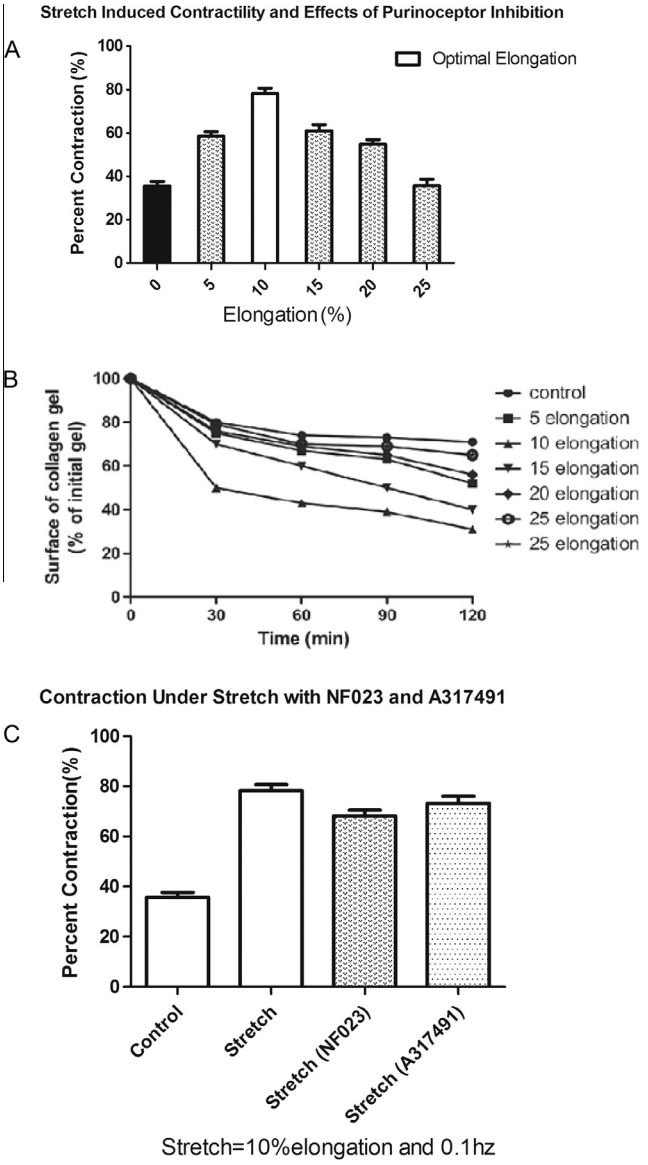

Fig. 2. Stretch induced contractile changes in bladder smooth muscle cells. Variation in magnitude of applied stretch caused contractile changes as seen in this figure. Maximum contractility was observed at 10% elongation as shown in (A). (B) Depicts the percentage of initial gel and subsequent contraction plotted versus time. While (C) shows effects of inhibitors on stretch modulated contractility for P2X1 (NF023) and P2X2 (A317491) respectively.

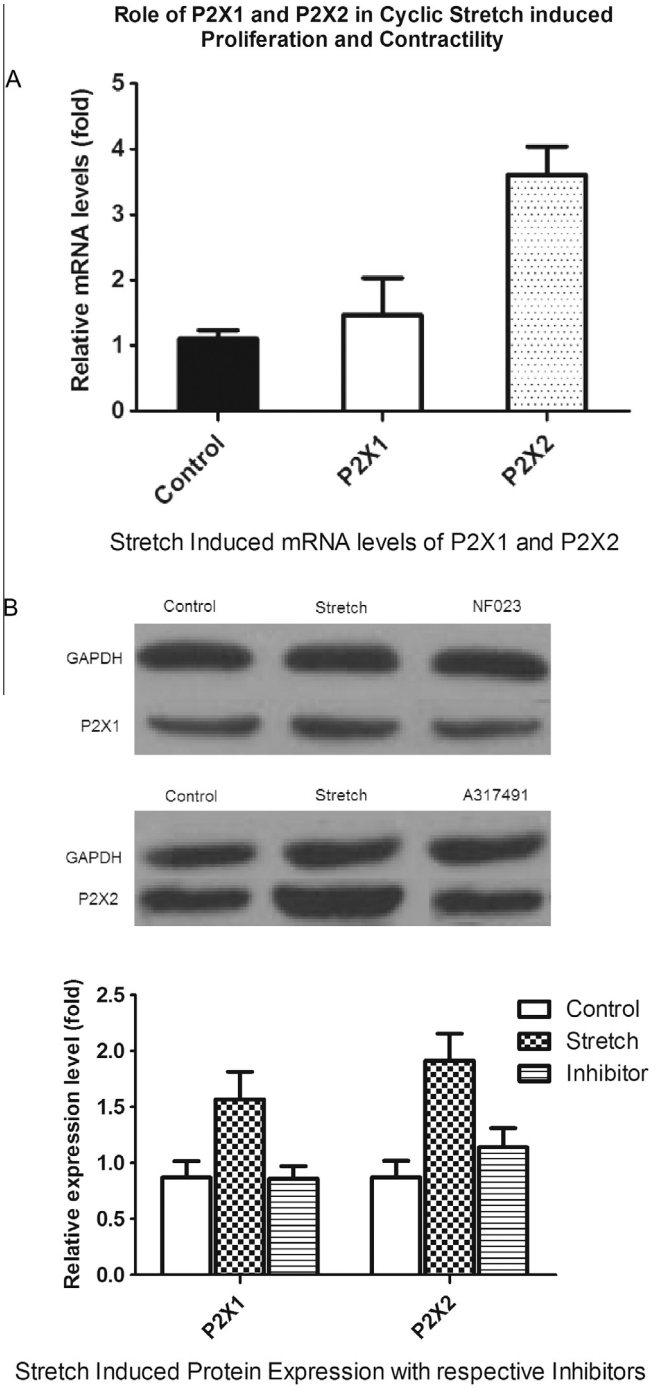

Fig. 3. Role of P2X1 and P2X2 in stretch induced changes of bladder smooth muscle cells. The P2X1 and P2X2 mRNA increased following application of stretch. Interestingly the increase is significantly larger for P2X2 as compared with P2X1. Expression of P2X1 and P2X2 increased (B and C). (B) Western blotting results from a typical study. (C) Densitometric quantification. Shown values are the mean + SD from three independent experiments.

for BSMC were obtained and these were used as control to investigate the role of purinoceptors. Although all seven types ($P2X_{1-7}$) have been reported in bladder; P2X1 and 2 are the major subtypes present on BSMC.

Bladder afferent activity is a function of pressure and volume [25]; therefore, the relationship of afferent activity to bladder volume is affected by compliance which in turn is affected by proliferating BSMC (wall thickness) and tonicity (contraction–relaxation). The optimum physiologic stretch we established in first part of experiment will thus ensure that role of P2X1 and 2 is better inferred in proliferation and contractility.

Interestingly, these are not independently governed processes and are affected by presence or absence of urothelium as contradictory results have been reported previously [26,27].

While P2X1 is the predominant subtype in BSMC, the expression profile of P2X2 is comparably bigger as seen in our results. Thus P2X2 homomer is possibly more susceptible to stretch related changes, resulting in diminished volume responsiveness and nociception.

Although a 3D recreation, compared with 2D environment in our experiment would have been more ideal. We believe that by providing optimum stretch conditions and obtaining functional role of purinoceptors in normal BSMC, the pathologic role can be better understood in conditions with atropine resistance; neurogenic and idiopathic detrusor over activity, bladder outlet obstruction, and Interstitial Cystitis/painful bladder syndrome.

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