

## Nicotine interferes with purinergic signaling in smooth muscle cells isolated from urinary bladders of patients with lower urinary tract symptoms

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**Abstract.** In patients with outlet obstruction, the contraction of the base is reduced compared to that of healthy individuals, while the contraction of the dome is not affected. Here, we investigated the cellular mechanisms that might be responsible for cholinergic effects blocking non-adrenergic non-cholinergic contractions in the base of the urinary bladder.

Smooth muscle cells either from the base or from the dome of human urinary bladders were cultured to determine the contribution of cholinergic and purinergic mechanisms to their Ca<sup>2+</sup> homeostasis.

While ATP evoked Ca<sup>2+</sup> transients in all the cells, nicotine and carbachol induced Ca<sup>2+</sup> transients only in 56% and 44% of the cells, respectively. When ATP was administered together with nicotine or carbachol, the amplitudes of the Ca<sup>2+</sup> transients recorded from cells prepared from the base of bladders were significantly smaller (42 ± 6% with nicotine and 56 ± 9% with carbachol) than those evoked by ATP alone. This inhibition was much less apparent in the dome of bladders.

The inhibition between the cholinergic and purinergic signaling pathways reported in this work may decrease the strength of the contraction of the base of the urinary bladder in patients with outlet obstruction during voiding.

**Key words:** Human urinary bladder — Smooth muscle — Nicotinic acetylcholine receptors — P2X purinergic receptors — Interaction

### Introduction

During aging, the human urinary bladder undergoes significant plasticity that contributes to the development of detrusor overactivity, impaired contractility, or the combination of both (Malone-Lee and Wahedna 1993; Yoshida et al. 2001). The originally flawless storage and emptying function of the bladder becomes altered, partly as a result of changes in bladder smooth muscle and connective tissue function, in addition to changes in afferent and efferent innervations

(Resnick and Yalla 1987). While 4-D ultrasound images of healthy human bladders show uniform contraction during voiding, the shortenings of the upper and lower parts of urinary bladders with outlet obstruction are different (Hirahara et al. 2006). Namely, during voiding only the dome shows strong contraction, whereas the shortening of the base is impaired (Hirahara et al. 2006). An interesting question is how the different parts of the urinary bladder can respond differently to the same stimulation.

During electrical stimulation of the parasympathetic nerves, acetylcholine (ACh) and adenosine triphosphate (ATP) are co-released from the nerve terminals. ACh and ATP stimulate muscarinic and purinergic receptors of the smooth muscle, respectively, and elicit bladder contraction (Dowdall et al. 1974; Kasakov and Burnstock 1982; Theobald and de Groat 1989). Although ATP plays an additional role

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in evoking the contraction of the detrusor in many species (rabbit, rat, mice; Calvert et al. 2001; Aronsson et al. 2010; Gomez-Pinilla et al. 2011), it has little, if any, role in healthy human bladders (Sjogren et al. 1982; Bayliss et al. 1999). On the other hand, the proportion of atropine-resistant, nerve-mediated contractions in human bladders increases in several pathological conditions (interstitial cystitis, idiopathic detrusor overactivity, etc.; Bayliss et al. 1999; Sjogren et al. 1982; O'Reilly et al. 2002). A possible explanation of the different phenotypes of contractions is that in bladders from pathological states, ectonucleotidase enzyme activity is reduced. Consequently, some of the released ATP is able to activate the detrusor and generate the atropine-resistant component of the contractions (Harvey et al. 2002).

It is known that co-activation of nicotinic and P2X<sub>2</sub> purinergic receptors inhibits the functions of both ion channels through direct protein-protein interaction on the neuronal cell membrane (Nakazawa 1994; Barajas-Lopez et al. 1998; Zhou and Galligan 1998; Khakh et al. 2000, 2005). Similar interaction between nicotinic and purinergic receptors in rat urinary bladder smooth muscle has been recently described (Jenes et al. 2012).

In this work, we investigated the cholinergic and purinergic contribution to the Ca<sup>2+</sup> homeostasis of smooth muscle cells isolated from human urinary bladders. We present evidence about the location dependence and suggest a possible physiological relevance of purinergic-cholinergic receptor cross-inhibition in a human non-neural tissue.

## Materials and Methods

### *Human urinary bladder smooth muscle cell culture*

Human bladder strips were prepared from histological specimens excised from patients undergoing bladder surgery due to various conditions (benign prostate hyperplasia, tumor of the prostate or bladder, nephrolith). The use of human detrusor specimens was approved by the Ethical Committee of the University of Debrecen. Informed consent was obtained from all subjects.

We investigated urinary bladder specimens from 12 male patients. The age of the patients was 69 ± 5 years (mean ± SD). Six patients had benign prostatic hyperplasia (BPH), three patients suffered from carcinoma of the urinary bladder, two patients had prostate cancer, and one had undergone surgery for stones in the bladder. Their International prostate symptom score (IPSS) ranged from 3 to 17, or it was not evaluated in patients who had urethral catheters.

There were two definite sites of the bladder where samples were taken from: one for the dome and one for the base. Anterior wall of bladders were cut open in the midsagittal plane. Dome samples originate from the anterior wall of the

dome, just from the wall next to the midsagittal incision. Base samples originate from the trigonum vesicae. The surgical procedure determined whether samples were collected from the dome only, from the base only, or from both the dome and the base.

Smooth muscle cells were cultured as described previously (Jenes et al. 2012), with an increased collagenase concentration to 1 mg/ml. Following removal of the mucosal layer the muscle piece was placed into trypsin (0.75 mg/ml) – EDTA (0.4 mg/ml) solution for 30 minutes at 37°C. After mechanical shredding, pieces were digested in RPMI 1640 solution containing 1 mg/ml collagenase (45 minutes at 37°C). Cells were washed in RPMI solution containing 10% FBS, then seeded onto glass coverslips. Experiments were carried out on 5–7-day-old cultures.

### *Fluorescent measurement of [Ca<sup>2+</sup>]<sub>i</sub>*

Changes in the concentration of [Ca<sup>2+</sup>]<sub>i</sub> were detected using the fluorescent dye Fura-2 as described in our previous reports (Bíró et al. 1998; Csernoch et al. 2000; Jenes et al. 2012). The coverslips with the Fura-2 loaded cells (90 min, 37°C, 15 μM Fura-2 AM) were placed on the stage of an inverted fluorescence microscope (Diaphot, Nikon, Japan). The measuring bath was constantly perfused with HEPES-buffered Tyrode's solution (in mM: 137 NaCl, 5.4 KCl, 0.5 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 11.8 HEPES-NaOH), supplemented with 1 g/l glucose (pH 7.4).

Following test solutions – dissolved in HEPES-buffered Tyrode's solution – were directly applied to the vicinity of the cells through a local perfusion capillary: ATP (180 μM), nicotine (5 μM) and carbachol (10 μM). Experiments were performed at room temperature (22–24°C). Excitation wavelength was altered between 340 and 380 nm, while the emission was monitored at 510 nm. Background fluorescence was measured at cell-free regions of the coverslip and subtracted automatically. [Ca<sup>2+</sup>]<sub>i</sub> levels were calculated according to the method of Grynkiewicz et al. (1985) from the ratio ( $R = F_{340}/F_{380}$ ) of the fluorescence intensities measured with excitation wavelengths 340 nm ( $F_{340}$ ) and 380 nm ( $F_{380}$ ) as previously described (Bíró et al. 1998; Csernoch et al. 2000) using *in vivo* calibration data.

Time derivative of [Ca<sup>2+</sup>]<sub>i</sub> was calculated and its maximum was taken as the maximum rate of rise. To determine the decay time constant, single exponential function was fitted – using the least squares method – to the declining phase of the calcium transient.

### *Immunocytochemistry*

Cultured smooth muscle cells were fixed in acetone for 5 min and permeabilized by 0.1% Triton X-100 (Sigma-Aldrich) in phosphate buffered saline (PBS, pH 7.4), for 10 min. After washing in PBS solution and blocking in 1%

bovine serum albumin (BSA, Sigma-Aldrich) for 30 min, cells were incubated with the primary antibodies: anti-P2X<sub>2</sub> (1:100), anti-nAChR $\alpha_3$  (1:200) and anti-nAChR $\alpha_7$  (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. Slides were washed three times in PBS and incubated with fluoresceine isothiocyanate- (FITC) conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) for 45 min (1:200). The nuclei of the cells were visualised using 4',6-diamidino-2-phenylindole (DAPI, Vector). Cells were examined on a confocal microscope (LSM 510 META, Zeiss, Oberkochen, Germany).

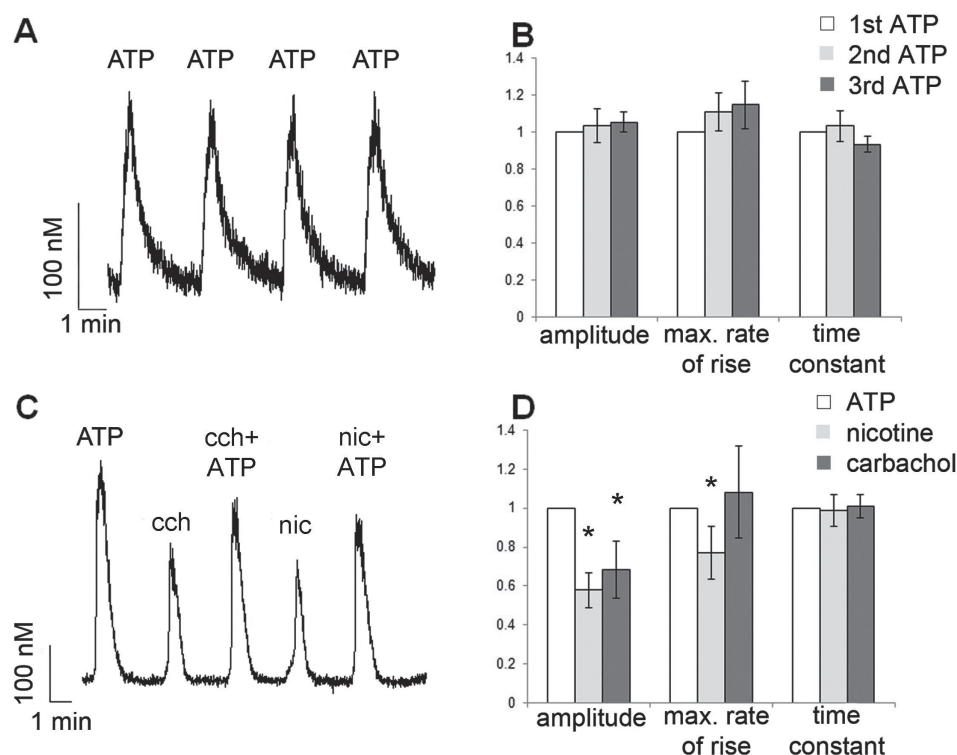
#### Immunohistochemistry

Tissue specimens were immediately fixed in 4% buffered formaldehyde (24 h), embedded in paraffin wax, and 4  $\mu$ m thick sections were cut. Endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub> in methanol (10 min, room temperature). Non-specific binding was prevented by incubating the sections with bovine serum albumin (BSA) or horse serum (HS, Sigma-Aldrich). Tissue sections were incubated

overnight at 4°C with anti-P2X<sub>2</sub>, anti-nAChR $\alpha_3$ , or anti-nAChR $\alpha_7$  primary antibodies (1:200). After incubation, the sections were rinsed three times with PBS. When the primary antibody raised in rabbit was used, the tissue sections were incubated with the EnVision system (Dako, Carpinteria, CA, USA), and visualization was achieved by employing the 3,3'-diaminobenzidine (DAB) SK4100 kit (Vector). When primary antibodies raised in goat were used (anti-P2X<sub>2</sub> and anti-nAChR $\alpha_7$ ), the sections were incubated in biotinylated anti-goat secondary antibodies for 30 min (1:100, Dako), rinsed with PBS and incubated with horseradish peroxidase-conjugated streptavidin (30 min; 1:500; room temperature). Visualisation of the immunolabelling was achieved using DAB. The sections were slightly counterstained with haematoxylin. Control experiments (sections incubated overnight without the primary antibodies) were regularly performed.

#### Statistics

Data obtained from any cell were normalized to the first ATP-evoked response of the same cell. The groups were



**Figure 1.** Calcium transients evoked by repetitive ATP applications or by cholinergic agonists on cultured smooth muscle cells of human urinary bladders. **A.** ATP-evoked (180  $\mu$ M) Ca<sup>2+</sup> transients in urinary bladder smooth muscle cells. **B.** Pooled data from 10 similar experiments where the main parameters (amplitude, maximal rate of rise, decay time constant) of the 2<sup>nd</sup> and 3<sup>rd</sup> ATP-evoked transients were normalized to those of the first response. **C.** Representative Ca<sup>2+</sup> transients induced by carbachol (cch, 10  $\mu$ M), nicotine (nic, 5  $\mu$ M), and/or ATP (180  $\mu$ M). **D.** Normalized parameters characterizing the Ca<sup>2+</sup> transients produced by the application of ATP, nicotine, or carbachol. \* indicates significant difference from the corresponding ATP-evoked parameter ( $p < 0.05$ ).

compared by Student's *t*-tests. Differences were regarded significant at  $p < 0.05$ . Data are expressed as mean  $\pm$  SEM.

## Results

### *Effect of the repetitive ATP application on the Ca<sup>2+</sup> transients of cultured smooth muscle cells from human urinary bladders*

To study the effects of the purinergic and cholinergic stimulation, cultured human urinary bladder smooth muscle cells were used. Throughout the experiments, all cells responded to ATP. First, the shape and reproducibility of the ATP-induced Ca<sup>2+</sup> transients were determined. ATP was applied for 40 seconds several times (with 3 minute intervals) to induce Ca<sup>2+</sup> transients by the activation of P2 receptors. There were no significant changes in any of the investigated parameters (amplitude, maximal rate of rise, decay time constant) of the consecutive Ca<sup>2+</sup> transients (Fig. 1A,B). In addition, no difference was observed between smooth muscle cells isolated from the base or the dome of the bladders.

### *Effects of nicotine and carbachol application on the Ca<sup>2+</sup> transients*

Nicotine and carbachol (5  $\mu$ M and 10  $\mu$ M, respectively) induced Ca<sup>2+</sup> transients in 30 cells from 54 cells investigated (56%) and 19 cells from 42 cells investigated (44%), respectively. The amplitude and the maximal rate of rise of the

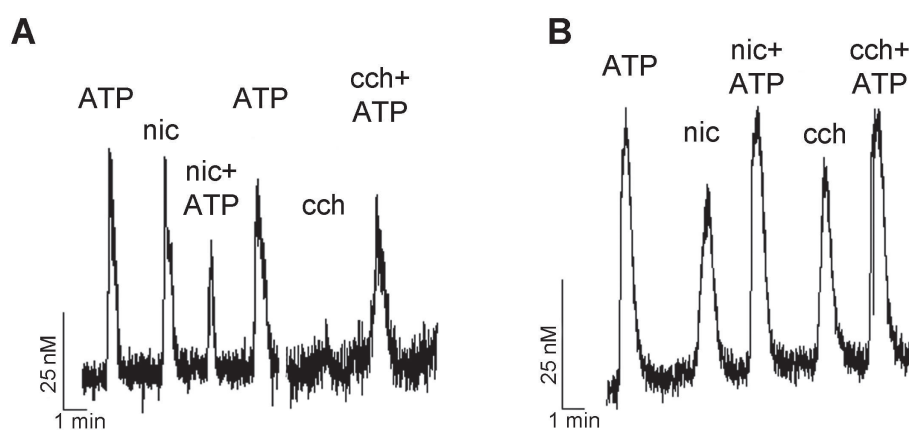
calcium transients evoked by either agonist were significantly lower than those of the responses evoked by ATP, but the decay time constants were the same (Fig. 1C,D). The effects of nicotine and carbachol were not significantly different from one another (Fig. 1D).

Cholinergic agonists (nicotine and/or carbachol) produced calcium transients in a lower proportion of cells from the base (in 22 cells from 42 cells tested, 52%) than from the dome (in 14 cells from 17 cells tested, 82%). The amplitudes of the responses, however, did not differ at all ( $58 \pm 7\%$  and  $54 \pm 8\%$  of the ATP-evoked responses in the base and in the dome, respectively).

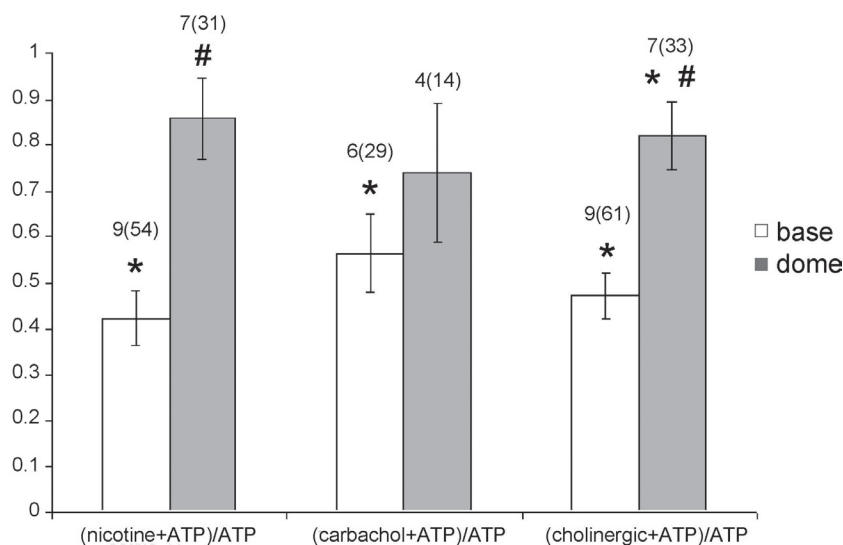
### *Interaction between purinergic and cholinergic pathways at the level of Ca<sup>2+</sup> transients of cultured smooth muscle cells isolated from the base of human urinary bladders*

The additive effect of ATP and the cholinergic agonist (nicotine or carbachol) was smaller than the sum of their effect individually (even smaller than the effect of ATP alone). In the base (Fig. 2A) the inhibition exhibited by nicotine was significantly more pronounced than in the dome (Fig. 2B) of the bladders.

In the base, nicotine massively reduced the amplitude of the ATP-evoked Ca<sup>2+</sup> transients (to  $42 \pm 6\%$ , in 54 cells, prepared from 9 patients), whereas the amplitude of the Ca<sup>2+</sup> transients evoked by ATP and carbachol were  $56 \pm 9\%$  of the ATP-evoked responses (in 29 cells, prepared from 6 patients), (Fig. 3). The responses to the combined application of ATP and the cholinergic agonist were  $47 \pm 5\%$  of the ATP-evoked responses (Fig. 3). In 22 cells where the effects



**Figure 2.** Representative calcium transients evoked by cholinergic and purinergic agonists on cultured smooth muscle cells from the base and from the dome of human urinary bladders. **A.** Representative Ca<sup>2+</sup> transients induced by purinergic, cholinergic, or combined purinergic-cholinergic stimulation of a smooth muscle cell isolated from the base of a urinary bladder. **B.** Representative Ca<sup>2+</sup> transients induced by purinergic, cholinergic, or combined purinergic-cholinergic stimulation of a smooth muscle cell isolated from the dome of a urinary bladder. The concentrations applied were 180  $\mu$ M, 10  $\mu$ M, 5  $\mu$ M for ATP, carbachol (cch) and nicotine (nic), respectively.



**Figure 3.** Amplitudes of the  $\text{Ca}^{2+}$  transients evoked by cholinergic and purinergic agonists on cultured smooth muscle cells. The amplitudes of the transients evoked by ATP in the presence of  $5 \mu\text{M}$  nicotine or  $10 \mu\text{M}$  carbachol were normalized to the amplitudes of the transients evoked by ATP alone. Each bar summarizes the effect of drug applications indicated underneath the X axis. The first number above each column indicates the number of patients, whereas the second number (in parentheses) gives the number of cells/drug applications investigated. \* indicates if the combined effect was significantly different from that of the ATP alone ( $p < 0.05$ ); # indicates significant difference between the responses of the base and of the dome ( $p < 0.05$ ).

of both nicotine and carbachol were tested, the former was usually more effective in reducing the amplitudes of the ATP-related transients.

In two patients, who had the mildest symptoms, and we obtained sample from the base (one patient with carcinoma transitiocellulare urinae, IPSS 6, and one patient with benign prostatic hyperplasia, IPSS 9), we found the cholinergic-purinergic interaction to be much less pronounced. In these cases, nicotine and carbachol, applied together with ATP, induced transients nearly the same amplitude ( $86 \pm 11\%$  and  $80 \pm 16\%$ , respectively) as ATP alone did.

#### *Effects of ATP, nicotine, and carbachol on the $\text{Ca}^{2+}$ transients of cultured smooth muscle cells from the dome of human urinary bladders*

The cross-reactivity between the purinergic and cholinergic receptors was also studied in smooth muscle cells ( $n = 33$ ) from the dome of urinary bladders cultured from 7 patients (Fig. 2B). Apparently, there was significantly less prominent cross-reactivity between the purinergic and cholinergic pathways in the cells from the dome of the bladders. When the ATP was administered together with either nicotine or carbachol, it induced similar  $\text{Ca}^{2+}$  transients than ATP did alone (Fig. 2B).

On smooth muscle cells cultured from the dome of urinary bladders, neither nicotine ( $86 \pm 9\%$ ,  $n = 31$ ) nor carbachol ( $74 \pm 15\%$ ,  $n = 14$ , from 4 patients) exerted sig-

nificant inhibition on the amplitudes of ATP-evoked  $\text{Ca}^{2+}$  transients (Fig. 3), although, a slight overall inhibition by the cholinergic agents was observed ( $82 \pm 8\%$ ).

#### *Expression of nicotinic and $\text{P2X}_2$ receptors in smooth muscle cell cultures and urinary bladder tissue sections*

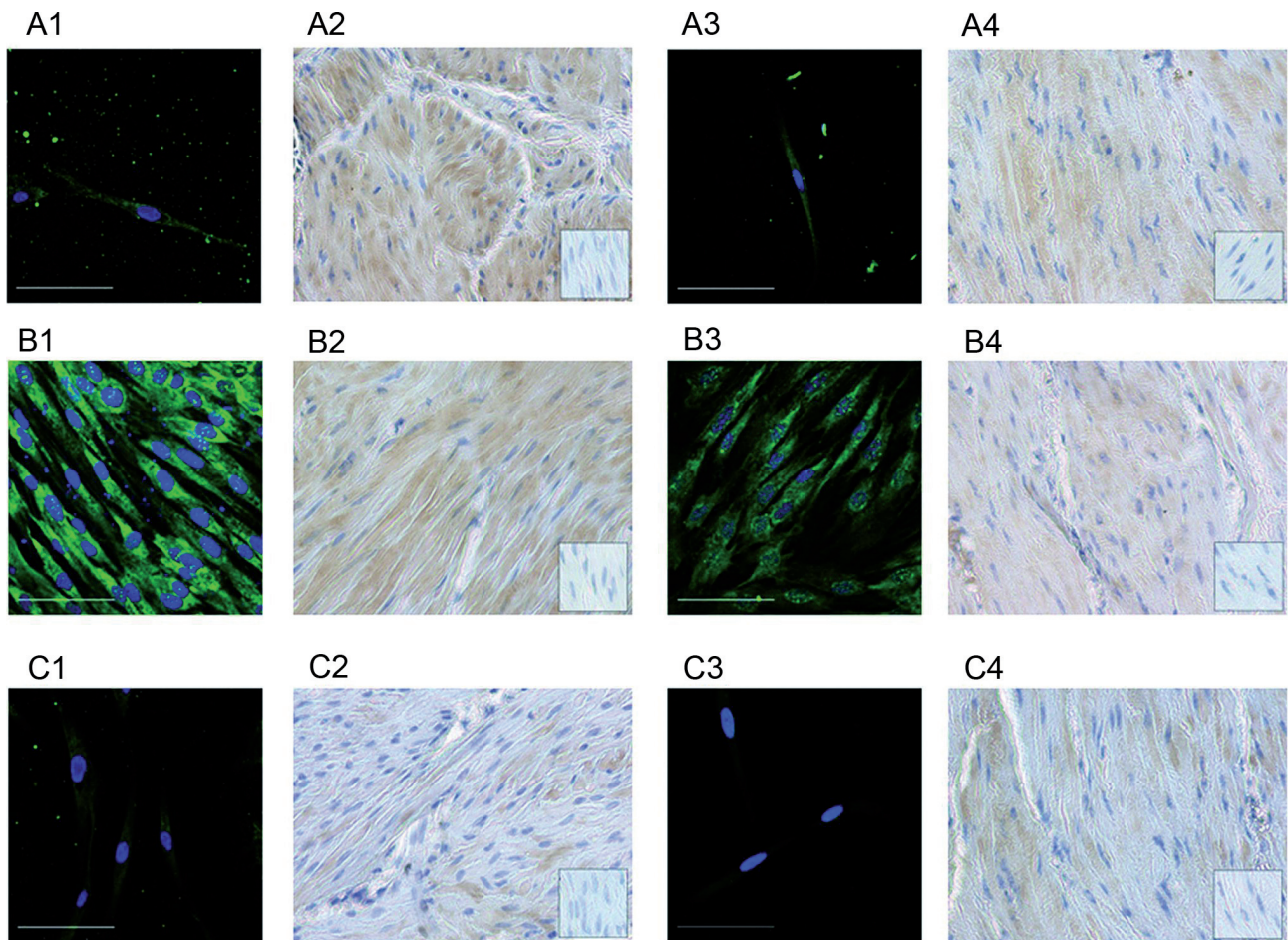
To determine the subtype-specific expression and distribution of the nicotinic receptors, cultured smooth muscle cells from human urinary bladders and bladder tissue sections were used. Cultured smooth muscle cells from the bladders showed  $\alpha_3$  and  $\alpha_7$  nicotinic acetylcholine receptor immunopositivity. The results of the immunohistochemistry experiments performed on tissue sections also confirmed the presence of both  $\alpha_3$  and  $\alpha_7$  nicotinic receptor subunits (Fig. 4).

The presence of  $\text{P2X}_2$  receptors was also demonstrated using both cultured cells and tissue sections. Both nicotinic receptor subunits ( $\alpha_3$  and  $\alpha_7$ ) and the  $\text{P2X}_2$  receptors showed similar distribution on the dome and on the base of all urinary bladder samples (Fig. 4).

## **Discussion**

This report presents evidence about the localization-dependence and suggests a possible physiological relevance of the cross-talk between purinergic and cholinergic receptors in





**Figure 4.** P2X<sub>2</sub>, nicotinic α<sub>7</sub>, and α<sub>3</sub> receptor-specific immunoreactivity of cultured human urinary bladder smooth muscle cells and histological tissue sections. A1–A4: Demonstration of the expression of nAChRα<sub>7</sub> receptor subunits. A1: cultured smooth muscle cells isolated from the dome; A2: histological tissue section prepared from the dome; A3: cultured smooth muscle cells isolated from the base; A4: histological tissue section prepared from the base. B1–B4: Expression of nAChRα<sub>3</sub> receptor subunits. The organization of the images is the same as in A1–A4. C1–C4: Expression of P2X<sub>2</sub> receptors. The organization of the images is the same as in A1–A4. On the immunocytochemistry images green colour represents the nAChRα<sub>7</sub> (A1, A3), nAChRα<sub>3</sub> (B1, B3), or P2X<sub>2</sub> receptor-specific (C1, C3) immunoreactivity, DAPI shows the cell nuclei. Calibration bars indicate 25 μm. The brown colour (DAB) in the immunohistochemistry images marks cells expressing nAChRα<sub>7</sub> (A2, A4), nAChRα<sub>3</sub> (B2, B4), or P2X<sub>2</sub> receptors (C2, C4). All sections were slightly counterstained with haematoxylin. Original magnification is ×200. Inserts show negative controls.

a non-neural tissue. We suggest that the inhibition between these signaling pathways is one of the underlying reasons of the inadequate contraction reported in the base of the urinary bladders of patients with outlet obstruction during voiding.

We demonstrated that nicotine and carbachol reduced – or even prevented – the ATP-evoked Ca<sup>2+</sup> transients in smooth muscle cells from urinary bladders. The cross-inhibition was more prominent in the base of the bladder. Similar cross-interaction was already described in rat smooth muscle cells from the base of the urinary bladder, when the smooth muscle cells were isolated from 8–12 weeks old animals (Jenes et al. 2012).

#### *Cholinergic and purinergic receptor activation of human urinary bladder smooth muscle cells*

Our results suggest interaction between P2X purinergic and a putative cholinergic receptor in detrusor. Previous experiments gave evidence on cholinergic and purinergic interaction between neuronal nicotinic and P2X<sub>2</sub> purinergic receptors in sympathetic ganglia (Nakazawa 1994), hippocampal neurons (Khakh et al. 2005), and myenteric neurons (Barajas-Lopez et al. 1998; Zhou and Galligan 1998; Brown and Galligan 2003). In these systems, co-application of a nicotinic and a purinergic agonist produced

smaller additive response (inward currents, calcium influx) than that was expected from independent receptor stimulations.

In our study both carbachol and nicotine were found to activate the acetylcholine receptors of the urinary bladder smooth muscle cells. The muscarinic receptor blocker atropine did not modify the purinergic-cholinergic interactions (13 cells from 2 patients). When we found cross-inhibition applying ATP and nicotine/carbachol together, atropine could not prevent this interaction, in addition, when such interaction was not observed, atropine did not invoke such interaction either. These data suggest that muscarinic acetylcholine receptors do not have any role in the purinergic-cholinergic receptor cross-inhibition. Among the nicotinic receptor subunits  $\alpha_1$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_7$ , and  $\alpha_9$  can be activated by both nicotine and carbachol (Alexander et al. 2008). However, the affinity of the  $\alpha_1$ ,  $\alpha_7$ , and  $\alpha_9$  subunits to carbachol is similar to, or even higher than that to nicotine. Since the inhibitory effect of carbachol on the ATP-evoked response was smaller ( $46 \pm 10\%$ ) than that of nicotine ( $71 \pm 7\%$ ), we imply that the  $\alpha_3$  nicotinic ACh receptor subunit is involved in the cross-talk with the purinergic receptors, however, the role of other subtypes can not be excluded.

Furthermore, the functional results clearly indicated that nicotinic receptors are present in the smooth muscle cells of the human bladder. It must be noted that although our immunohistochemistry data suggested the presence of  $\alpha_7$  nicotinic receptor subunits in human bladders, it is unlikely that this subunit alone is responsible for mediating the effects of cholinergic stimulation, as it was reported in rat urinary bladders (Jenes et al. 2012). The possible roles of other nicotinic receptor subunits are suggested by the marked difference between the cholinergic agonists that are capable of interfering with purinergic stimulation in rats and humans. More specifically, in rats only carbachol inhibits the ATP-evoked transients, whereas nicotine does not. In contrast, in the human bladder nicotine was also effective and it inhibited the ATP-evoked transients at least as effectively as carbachol did. In line with the implication of the significance of other nicotinic receptor subunits, our immunostaining data indicated the presence of  $\alpha_3$  nicotinic receptor both in cultures and urinary bladder tissue samples. Our study, therefore, provides evidence that nicotinic receptors are functionally present in the bladder smooth muscle. Additionally, we demonstrated that nicotinic receptors may play an important role in modulating the activity of purinergic receptors.

It seems probable that among purinergic receptors, P2X<sub>2</sub> receptors have importance in the cholinergic-purinergic interaction in human bladders. The observation that the ATP-evoked Ca<sup>2+</sup> transients displayed no desensitization is an important piece of evidence corroborating the hypothesis about the functional relevance of P2X<sub>2</sub> receptors, especially

as they are known to desensitize slowly and recover rapidly (North 2002). In fact, our immunocytochemistry and immunohistochemistry data also indicated the presence of P2X<sub>2</sub> receptors. It has been noted that both P2X<sub>2</sub> receptor expression and the consequent purinergic component of detrusor contractions are significantly elevated in patients with idiopathic detrusor instability (O'Reilly et al. 2002). We propose, therefore, that cholinergic modulation of the P2X<sub>2</sub> receptors may be behind the impaired bladder function of these patients.

It has to be mentioned, that our patients had lower urinary tract symptoms with different severity, and we have no information about a possible cholinergic-purinergic interaction in healthy human bladders. Nevertheless, this inhibition seems to be more pronounced in our patients with more severe symptoms (higher International Prostate Symptom Score, larger post-void residual volume, usually complete urinary retention). On the other hand, patients with complete urinary retention had urethral catheters, and the possibility that catheters might influence the observed interaction cannot be excluded.

**Acknowledgements.** This work was supported by grants from the Hungarian Scientific Research Fund (OTKA F68393, K75604), Hungarian Ministry of Health (ETT 186/2009), Hungarian Ministry of Education (TÁMOP-4.2.2-08/2008-0019), the European Union and the European Social Fund (TÁMOP-4.2.2.A-11/1/KONV-2012-0025). The authors are indebted for the excellent technical assistance from R. Öri and I. Kiss.

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Received: October 29, 2012

Final version accepted: January 25, 2013