A POSSIBLE ROLE OF THE CHOLINERGIC AND PURINERGIC RECEPTOR INTERACTION IN THE REGULATION OF THE RAT URINARY BLADDER FUNCTION

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Abstract

Under pathological conditions the shortening of the upper (dome) and lower (base) parts of the urinary bladder are different. Namely, the contraction of the base is milder than that of the dome. To understand how different parts of the urinary bladder could respond differently to the same stimulation, the cellular mechanisms that might be responsible for cholinergic effects blocking non-adrenergic non-cholinergic contractions in the rat urinary bladder were investigated. Rat urinary bladder smooth muscle cells were thus freshly isolated or cultured and the contribution from cholinergic and purinergic pathways to their Ca^{2+} homeostasis together with the expression of nicotinic (nAChR) and purinergic receptors was examined. The ATP-evoked Ca^{2+} transients in rat smooth muscle cells did not show any desensitization. However, when ATP was administered together with carbamylcholine (CCh), the latter essentially prevented ATP from evoking Ca^{2+} transients in smooth muscle cells from the base (suppression to 12±2.5% of control, n=57; $p<0.01$), but not from the dome (99±5% of control, n=52; $p>0.05$) of the rat urinary bladder. While atropine was unable to modify (6±3% of control, n=14; $p<0.05$), α-bungarotoxin (118±12% of control, n=20; $p>0.05$) blocked the inhibitory effects of CCh. Additionally, α7 subunits of nAChR were identified using immunocytochemistry, immunohistochemistry, and Western blot in cultured urinary bladder smooth muscle cells, in urinary bladder sections, and in urinary bladder muscle strips, respectively, suggesting that the activation of nAChR modifies the action of ATP.

Keywords

non-adrenergic, non-cholinergic contractions; nicotinic acetylcholine receptors; purinergic receptors; receptor interaction; urinary bladder smooth muscle

Short title

Cholinergic-purinergic receptor interaction in rat bladder smooth muscle

Introduction

The function of the urinary bladder is to relax and store urine during filling, and contract to empty the bladder during micturition. These roles are achieved through complex interactions between the autonomic nervous system and the urinary bladder, primarily the smooth muscle cells. Urinary bladder smooth muscle in adult rats is well innervated (Gabella, 1999). The bundles of nerve fibres branch repeatedly in urinary bladder smooth muscle and eventually become single fibres containing varicosities. The varicosities contain neurotransmitters, including acetylcholine (ACh) and adenosine triphosphate (ATP) (Gabella, 1995).

During electrical stimulation of the parasympathic nerves ACh and ATP are co-released from the nerve terminals to stimulate the muscarinic and purinergic receptors of the smooth muscle cells, respectively, and to elicit bladder contraction (Dowdall *et al.* 1974; Kasakov and Burnstock, 1982; Theobald and de Groat, 1989). Acetylcholine is a ubiquitous transmitter, while ATP plays an additional role in many species, including rats (Calvert *et al.* 2001; Somogyi *et al.* 2002; Szigeti *et al.* 2005). Acetylcholine acts via M_3 receptor activation, generation of intracellular inositol-1,4,5-trisphosphate (IP_3) and Ca^{2+} release from intracellular stores. There is also evidence that muscarinic activation leads to Ca^{2+} influx (Schneider et al., 2004). Conversely, ATP binds to ionotropic P2X receptors, depolarizing the cell membrane and instigating Ca^{2+} influx through voltage-activated (mainly L-type) Ca^{2+} channels (Andersson and Arner, 2004). The Ca^{2+} influx could initiate Ca^{2+}-induced Ca^{2+} release from intracellular stores and lead to contractile protein activation. Thus, modulation of intracellular calcium concentration ($[Ca^{2+}]_i$) remains a common pathway to regulate detrusor contractility, with additional modulation via alterations to the Ca^{2+} sensitivity of contractile proteins (Andersson and Arner, 2004; Arner *et al.* 2007).

Transient increases in $[Ca^{2+}]_i$ in smooth muscle have been recorded following the activation of purinergic receptors. In urinary bladder smooth muscle, the activation of purinergic receptors may induce excitatory junctional potentials and Ca^{2+} transients (Bramich and Brading, 1996; Hashitani *et al.* 2000). Field stimulation of the smooth muscle evoked local, transient Ca^{2+} release events. These events were mediated by postsynaptic smooth muscle P2X receptors activated by ATP released from synaptic varicosities (Brain *et al.* 2002, 2003). Pharmacological studies also confirmed that junctional Ca^{2+} transients were mediated through the activation of purinergic receptors (Lamont and Wier, 2002; Lamont *et al.* 2003).

Ligand-gated receptors may interact and regulate one other both at the neuronal and the end-organ levels and modulate their responses to external stimuli (Searl and Silinsky, 1998). G-protein coupled metabotropic receptors commonly "cross-talk" through intracellular second messenger molecules and protein phosphorylation (de Blasi et al., 2001; Moldrich and Beart, 2003). Recently, ionotropic receptors have also been shown to interact directly with one another in ganglia. Co-activation of the nicotinic and the $P2X_2$ purinergic receptors inhibited the functions of both ion-channel receptors through direct protein-protein interaction on the neuronal cell membrane (Nakazawa, 1994; Searl et al., 1998; Zhou and Galligan, 1998; Barajas-Lopez et al., 1998; Khakh et al., 2000, 2005). Negative interactions between the nicotinic and 5-HT_3 receptors (Barajas-Lopez et al., 2002; Boue-Grabot et al., 2003) and between the $GABA_A$ and P2X purinergic receptors (Sokolova et al., 2001) have also been described when the two ionotropic receptors were co-activated in neurons. However, similar interaction between nicotinic and purinergic receptors at the bladder smooth muscle level has not been described on cellular but suggested at tissue level (Lai *et al.* 2008).

Here we investigated the cholinergic and purinergic contribution to the Ca^{2+} homeostasis of smooth muscle cells isolated from young adult rat urinary bladders. Given the potential role for nicotinic acetylcholine receptors (nAChRs) in the neural control of bladder function, the current study was aimed to determine whether nAChRs are also expressed in the smooth muscle cells and whether activation of these receptors can alter the function of these cells. We present evidence for the existence and determine its location dependence of the interaction between the purinergic and cholinergic signaling in smooth muscle cells of the urinary bladder. Certain aspects of these results were presented at the Annual Conference of the European Muscle Society (Jenes *et al*. 2006; Szigeti *et al*, 2007).

Materials and Methods

All experiments were performed in accordance with Hungarian guidelines and were in compliance with the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Council of Europe No 123, Strasbourg 1985).

Smooth muscle cell culture from rat urinary bladder

Young adult Wistar rats (male and female, 8-12 weeks old) were terminally anaesthetized by isoflurane, bladders were removed, opened and cut into a base and a dome part. The pieces were placed into Hank's solution and the urothelial layer was peeled off. Each smooth muscle piece was placed into 5 ml 0.75 mg ml-1 trypsin (Sigma-Aldrich, Heidelberg, Germany)-0.4 mg ml-1 EDTA (Sigma-Aldrich, Heidelberg, Germany) solution, and incubated in a shaking bath for 30 minutes at 37 °C. After transferring into RPMI 1640 (Roswell Park Memorial Institute) solution containing 0.75 mg ml-1 collagenase (Sigma-Aldrich, Heidelberg, Germany) the smooth muscle was cut into small pieces and incubated in shaking bath for further 45 minutes at 37 °C. After incubation 2 ml fetal bovine serum (FBS) was added, cells were centrifuged at 1000 RPM for 10 minutes, then the superfusate containing collagenase was removed. Cells were resuspended in 3 ml RPMI solution containing 10% FBS and centrifuged again at 1000 RPM for 10 minutes (twice). After the last cleaning process 3 ml RPMI solution containing 10% FBS was added for 3 plates (diameter 3 cm) having 1 ml cell suspension each. This medium was renewed every second day until the cells reached a confluence of 60-70 %. At that point, the FBS content of the RPMI medium was reduced to 1% for 2-3 more days to facilitate myocyte differentiation. Experiments were carried out on 5-7-days-old cultures.

The smooth muscle cells in the cultures were morphologically distinct from the interstitial cells and their identity were confirmed with immunocytochemistry using primary antibodies against alpha smooth muscle actin.

Freshly isolated smooth muscle cells from rat urinary bladder

Same method was used as for the cell culture, but the isolated cells were tested right after they attached to the coverslips. All of these experiments were carried out within 20 hours after removing the urinary bladder from the rats.

Fluorescent measurement of $[Ca^{2+}]_i$

Changes in the concentration of $[Ca^{2+}]_i$ were detected using the fluorescent dye Fura-2 as described in our previous reports (Bíró *et al.*, 1998; Csernoch *et al*., 2000). To introduce the calcium-sensitive probe into the intracellular space cells were incubated for 90 min at 37 °C with 15 µM Fura-2 AM (Molecular Probes, Eugene, OR, USA). Coverslips were then washed with normal HEPES Tyrode's solution (in mM: 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 11.8 HEPES-NaOH, 1 g l-1 glucose, pH 7.4) and this solution also provided the extracellular environment for the cells throughout the entire measurement. The coverslips with the Fura-2 loaded cells were then placed on the stage of an inverted fluorescence microscope (Diaphot, Nikon, Japan). The measuring bath was constantly perfused with normal HEPES Tyrode's solution at a 2 ml min-1 rate (Econo Pump, Bio-Rad Hungary, Budapest, Hungary). Test solutions were directly applied to the cells through a local perfusion capillary (Perfusion Pencil™, AutoMate Scientific, San Francisco, CA, USA) with an internal diameter of 250 µm at a 1.5 µl s-1 rate, using a local perfusion system (Valve Bank™8 version 2.0, AutoMate Scientific, San Francisco, CA, USA). Experiments were performed at room temperature (22-24°C). Excitation wavelength was altered between 340 and 380 nm by a dual-wavelength monochromator and an on-line connected microcomputer (Deltascan, Photon Technology International, New Brunswick, NJ, USA) while the emission was monitored at 510 nm using a photomultiplier at an acquisition rate of 10 Hz per ratio. Background fluorescence was measured at cell-free regions of the coverslip and subtracted automatically.

$[Ca^{2+}]_i$ 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.

Immunocytochemistry

Smooth muscle cells from urinary bladders were cultured on sterile plates. The 5-7 days old cells were fixed in acetone for 5 min and then permeabilized by 0.1% Triton-X-100 (Sigma-Aldrich, Heidelberg, Germany) in a phosphate buffered saline (PBS, 0.02 M NaH_2PO_4, 0.1 M NaCl) for 10 min. After washing with PBS solution and blocking with 1% bovine serum albumin (BSA, Sigma-Aldrich, Heidelberg, Germany) in PBS for 30 min, cells were incubated with anti-nAChRα7 primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-

$P2X_2$ receptor primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C (dilution 1:50, 1:200, 1:500). Plates were washed three times in PBS then incubated with fluoresceine isothiocyanate (FITC) conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) for 45 min (dilution 1:200). The nuclei of cells were visualised using 4',6-diamidino-2-phenylindole (DAPI). Cells were examined on a confocal microscope (Zeiss LSM 510 META, Zeiss, Oberkochen, Germany).

Immunohistochemistry

The tissue specimens were immediately fixed in 4% buffered formaldehyde (24 h), embedded in paraffin wax, and 4 μm thick sections were cut. The endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol (10 min, room temperature). Non-specific binding was prevented by incubating the sections with BSA. The tissue sections were then incubated overnight at 4 °C with anti-nAChRα7-specific primary antibodies (dilution 1:200, Santa Cruz Biotechnology). After incubation, the slices were rinsed three times with PBS for 5 minutes. The sections were incubated in biotinylated anti-goat secondary antibodies (raised in rabbit) for 30 min (1:100, Dako, Glostrup, Denmark), then rinsed with PBS and incubated with horseradish peroxidase-conjugated streptavidin (30 min; 1:500; room temperature). In these instances visualisation of the immunolabelling was also achieved using 3,3'-diaminobenzidine (DAB). At the end of the procedure slight counterstaining was performed using hematoxylin. Control experiments were regularly performed. In these cases, the sections were incubated overnight without the primary antibody.

Western blot

To determine the expression of nAChRα7 subunits in different parts of the young adult rat bladder, Western blot technique was applied. Tissues were homogenized in homogenization buffer [20 mM Tris-Cl, pH 7.4, 5 mM EGTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 μM leupeptin, all from Sigma] and the protein content of samples was measured by a modified bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% gels were loaded with 20 to 30 μg protein per lane), transferred to nitrocellulose membranes (Bio-Rad, Vienna, Austria), and then probed with the above mentioned anti-nAChRα7 antibodies (1:100). A horseradish peroxidase-conjugated rabbit anti-goat IgG antibody (1:500, Bio-Rad) was used as a secondary antibody, and the immunoreactive bands were visualized by

enhanced chemiluminescence (Gel Logic 1500 Imaging System, Carestream, Rochester, NY, USA). Western blotting experiments were repeated five separate times, and the protein bands were analyzed by Image J software provided by the NIH. The densitometric values were normalized to the values of actin immunoreactivity to correct any loading and transfer differences between samples.

Data analysis

Data were analyzed using Microsoft Excel® (Microsoft, Redmond, WA, USA) and Microcal Origin® (OriginLab, MA, USA) computer softwares. Data are expressed as mean±SEM. Student's t-test was used to compare paired data between the control group (first ATP application) and the other groups (purinergic and cholinergic agonists and antagonists), and 2-tailed $p<0.05$ was taken to indicate statistical significance.

Results

Effect of repetitive ATP application on the Ca^{2+} transients of cultured smooth muscle cells from the urinary bladder

To study the role of the purinergic and cholinergic stimulation, first the shape and reproducibility of the ATP-induced Ca^{2+} transients were determined on cultured rat urinary bladder smooth muscle cells (Fig. 1). ATP (180 μM) was applied for 40 seconds three times (at time zero, then three and six minutes later) to activate P2 receptors and induce Ca^{2+} transients. There were no visible changes in the second and third Ca^{2+} transients as compared to the first one (Fig. 1A). To quantify any possible changes that might have occurred, single exponential functions were fitted to the declining phase of the calcium transients and their time constant (τ) was determined. In addition, the peak of the first time derivative of the signal was used to assess its maximal rate-of-rise. Pooled data from 7 independent experiments (7 cells from 7 animals) confirmed that the amplitude (Fig. 1B), the τ (Fig. 1C) and the maximal rate-of-rise (Fig. 1D) of the second and third transients were not significantly different statistically ($p>0.05$) from those of the first transient.

Interaction between purinergic and cholinergic pathways at the level of the Ca^{2+} transients on freshly isolated and cultured smooth muscle cells

To understand the possible cross-reactivity between the purinergic and cholinergic signaling pathways, cultured and freshly isolated smooth muscle cells from adult rat urinary bladders were stimulated by the agonists of the two pathways (Fig. 2). While ATP was essentially always (more than 90% of the cells) capable of initiating a calcium transient, the parasympathetic agonists carbamylcholine (carbachol; CCh) and nicotine (nic) could not induce elevations in $[Ca^{2+}]_i$ in rat smooth muscle cells, either cultured (n=109) or freshly isolated (n=5).

However, when ATP was administered together with 10 μM CCh, CCh prevented ATP from evoking Ca^{2+} transients in the rat smooth muscle cell from the base of the urinary bladder (Fig. 2B, 2C). All together 57 cultured (Fig. 2B) and 3 freshly isolated (Fig. 2C) cells from the base of the urinary bladder prepared from 18 and 3, respectively animals were tested. While CCh decreased the amplitude of the ATP-evoked Ca^{2+} transients by 88±2.5% ($p<0.01$), this cross-inhibition was not present when ATP was applied together with 5 μM nicotine (Fig. 2). The effect of nicotine was tested on 20 cells obtained from 6 animals. On the other hand, neither carbachol nor

nicotine inhibited the effect of ATP in the cells of the dome of the urinary bladder (52 cells prepared from 16 rats, Fig. 2A).

Pharmacological characterization of the cross-inhibition

To establish the cholinergic receptor subtype responsible for the observed cross-inhibition in the cells of the base of the urinary bladders, muscarinic and nicotinic antagonists were applied (Fig. 3). The muscarinic receptor blocker atropine (1 μM; Fig. 3A, 3B) was unable to modify the purinergic-cholinergic interactions (24 cells from 4 animals). That is, on the one hand, when the cross-inhibition was present for ATP and CCh – on cells from the base –, the co-application of atropine with ATP and CCh could not prevent the purinergic-cholinergic inhibition (Fig. 3A, 4). On the other hand, when there was no interaction between the purinergic and cholinergic pathways without atropine – on cells from the dome –, there was no interaction between them in the presence of atropine either (Fig. 3B, 4).

In contrast, the presence of α-bungarotoxin – a specific competitive antagonist of the nicotinic acetylcholine receptor (nAChR) – prevented carbachol from interfering with the ATP-evoked calcium transients (Fig. 3C, 3D, 4). In 20 cells from 3 animals isolated from the base of urinary bladders CCh was unable to block the effect of ATP when α-bungarotoxin (500 nM) was present in the bathing medium (Fig. 3C, 4). Nevertheless, α-bungarotoxin did not interfere with the ATP-evoked signals since the simultaneous presence of CCh and the toxin did not modify the calcium transients in the cells isolated from the dome (Fig. 4D). The pooled data (Fig. 4) confirmed that while the effect of α-bungarotoxin was statistically significant ($p<0.05$) in cells from the base, the toxin did not modify significantly ($p>0.05$) the changes in $[Ca^{2+}]_i$ in cells from the dome.

To narrow the possible receptor candidates for the purinergic pathway different purinergic agonists were also tested. From the P2X receptor agonists neither α,β-methylene-ATP (10 μM; cultures from 6 animals, data from 25 cells, 13 cells from the base and 12 cells from the dome), nor 2-methyl-tio-ATP (180 μM; cultures from 3 animals, data from 7 cells, 4 cells from the base and 3 cells from the dome) induced any elevation in $[Ca^{2+}]_i$ (Fig. 5A). In this respect, there were no significant differences between the cells isolated from the base and the dome. In addition, the P2Y receptor agonist UTP (180 μM; cultures from 5 animals, data from 29 cells, 20 cells from the base and 9 cells from the dome) could not induce Ca^{2+} transients in cultured smooth muscle cells of the urinary bladder (Fig. 5B). Again, there were no significant differences, in this respect, between the cells from the

base and the dome. In addition, the L-type calcium channel blocker nifedipine (10 μM) significantly – to 38±15% of the response measured in the absence of nifedipine ($p<0.05$; 9 cells from 2 animals) – reduced the ATP-induced Ca^{2+} transients (data not shown). Noteworthy, that CCh was still able to further reduce the effect of ATP in the presence of nifedipine (to 0%; 2 cells from 1 animal).

Immunostaining of the nicotinic receptors and $P2X_2$ receptors in smooth muscle cell cultures and urinary bladder tissue sections

CCh can serve as an agonist for the α_1, α_3, α_4, α_7, and α_9 nAChR subunits and, furthermore, it has a higher affinity than nicotine does towards the α_1, α_7, and α_9 subunits (Alexander *et al.* 2008). This, together with the effects seen with α-bungarotoxin (see above) led us to suspect the involvement of the α_7 subunit of nAChR in the cross-inhibition detail before. To determine the distribution of the α subunit of the nicotinic receptors, cultured smooth muscle cells from rat urinary bladders (Fig. 6A) and bladder tissue sections (Fig. 6B) were used. Cultured smooth muscle cells from the rat urinary bladder, both from the base and from the dome, displayed α7 nicotinic receptor positivity, however, it seemed to be more intensive for cells from the base than that of the cells from the dome (Fig. 6A). The α7 nicotinic receptor immunoreactions in urinary bladder tissue sections showed similar distribution (Fig. 6B). Western-blot analysis confirmed the expression of nAChRα7 protein in rat urinary bladder smooth muscle strips (Fig. 6C). Furthermore, quantitative evaluation of the Western blots (normalizing the absorbance of the nAChRα7 band to that of the actin band) confirmed that the expression of nAChRα7 in the cells from the base was significantly higher than the expression in the cells from the dome (Fig. 6D, $p<0.05$).

Based on previous reports (Creed *et al.*, 2010; Dutton *et al.*, 1999; Lee *et al.*, 2000; Studeny *et al.*, 2005) and the pharmacological profile of the ATP-evoked calcium transients presented above, we suspected the presence of $P2X_2$ receptors in the rat urinary bladder smooth muscle. This was indeed confirmed using immunocytochemical and immunohistochemical surveys (Fig. 7). Distribution of the $P2X_2$ receptors appears to be rather cytoplasmic than membrane, which is in agreement with previous studies ().

Discussion

This report presents evidence of purinergic-cholinergic receptor cross-inhibition in non-neural cells. This cross-inhibition between the two signaling pathways was observed in the base, but not in the dome of the rat urinary bladder.

We demonstrated the contribution of the purinergic signaling pathway to the Ca^{2+} homeostasis in cultured rat smooth muscle cells. ATP was able to evoke Ca^{2+} transients in the smooth muscle cells and these transients did not show any desensitization. When ATP was administered together with the parasympathetic agonist carbachol, the latter prevented ATP from evoking Ca^{2+} transients in the cells from the base of the urinary bladder. Since α-bungarotoxin but not atropine was able to interfere with this interaction and because certain nicotinic receptors are also sensitive to CCh, we suspected the presence of the nAChRα7 on our cells. Smooth muscle cells from the rat urinary bladder – either isolated or *in situ* – displayed α7 nicotinic receptor positivity. Furthermore, cells from the base of the bladder seemed to have stronger immunopositivity than the cells from the dome as confirmed by Western blotting.

Cholinergic and purinergic receptor activation in cultured smooth muscle cells from the urinary bladder

Our results suggest a unique interaction between P2X purinergic and a putative nicotinic receptor in the bladder smooth muscle. Earlier measurements gave evidence on cholinergic (nicotinic) and purinergic interaction between neuronal type nicotinic and $P2X_2$ purinergic receptors in rat sympathetic ganglia (Nakazawa, 1994), hippocampal neurons (Khakh *et al.*, 2005), autonomic ganglia (Searl *et al.*, 1998), and myenteric neurons (Barajas-Lopez *et al.*, 1998; Zhou and Galligan, 1998; Brown and Galligan, 2003). In these systems, co-application of a nicotinic agonist (nicotine, cystine) and a purinergic agonist (ATP, α,β-methyleneATP) produced lower additive response (inward currents, calcium influx) then what was expected from independent receptor stimulations.

The question then was, whether there are nicotinic receptors present on urinary bladder smooth muscle cells. Traditionally smooth muscle is not considered to be equipped with nicotinic receptors (Jensen *et al.* 2005). It is important to note that even if nicotinic receptors are present in the bladder smooth muscle, they are silent since nicotinic agonists do not elicit a contraction and no intramural ganglia can be found in rat bladders (Alian and

Gabella, 1996; Somogyi and de Groat, 1992). However, expression of several isoforms of the nicotinic receptor has been demonstrated in vascular smooth muscle (Brüggmann et al., 2002). Nicotinic receptors are also found in the urothelium in urinary bladder (Beckel et al. 2006; Birder and de Groat, 2007). Our study provides evidence that while nicotinic receptors are present on the bladder smooth muscle cells, too, their activation is indeed not accompanied by an elevation in intracellular Ca^{2+} concentration. The current study, nevertheless, demonstrated that nicotinic receptors on the smooth muscle may play an important role in modulating the activity of the purinergic signal transduction.

Most of the experiments presented in this study were carried out on cells held in primary culture. Due to the fact that culturing could lead to alterations in receptor expression pattern or in the signaling pathways, we carried out similar experiments on freshly isolated cells, too. Since these cells responded similarly in all aspect – while carbachol was unable to generate Ca^{2+} transients it interfered with ATP in evoking an increase in $[Ca^{2+}]_i$ – we can conclude that the observations were not exclusively due to culturing.

The possible receptor subtype candidates for the cholinergic-purinergic receptor interaction

Based on our data on the pharmacology of the urinary bladder smooth muscle cells we suggest that the $P2X_2$ receptors have importance in this cholinergic-purinergic interaction. The P2 receptors displayed no or just a slow and partial desensitization. They were not activated by the application of α,β-methylene-ATP, 2-methyl-thio-ATP (data not shown) and UTP, consistent with the idea that ATP-induced Ca^{2+} transients are generated via activating $P2X_2$ receptors in the cultured urinary bladder smooth muscle cells. The presence of $P2X_2$ receptors on these cells has already been described (Creed *et al.*, 2010; Dutton *et al.*, 1999; Lee *et al.*, 2000; Studeny *et al.*, 2005) and were also detected through immunostaining in this study.

In our experiments the muscarinic receptor blocker atropine did not modify the purinergic-cholinergic interactions. When we found cross-inhibition applying ATP and CCh 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 acethylcholine receptors do not have any role in the purinergic-cholinergic receptor cross-inhibition. On the other hand, the presence of α-bungarotoxin prevented CCh from interfering with the purinergic signaling pathway on cells from the base but had no effect on cells from the dome. Since α-bungarotoxin is considered as a specific antagonist for nAChR, especially for the α7 subunit (Couturier *et al.*, 1990;

Kempsill *et al.*, 1999), we must assume a role of the nicotinic receptors instead. Our immunocyto- and immunohistochemistry, as well as Western-blot data confirmed the presence of nAChRα7 positive smooth muscle cells in cultures and in urinary bladder tissues from rat. It should be noted, however, that the muscarinic agonist carbachol had modulatory effect on the nicotinic acethylcholine receptors, while nicotine itself did not affect the inhibitory function of these receptors. Nevertheless, our results are in agreement with the findings that α_1, α_3, α_4, α_7, and α_9 nAChRs can be activated by carbachol (Alexander *et al.* 2008), moreover, the affinity of the α_1, α_7, and α_9 subunits to carbachol is higher than that to nicotine.

The functional relevance of the nicotinic-purinergic cross-inhibition in urinary bladder

Another observation in our study is that there is a difference in the receptor cross-inhibition between the base and the dome of the urinary bladder. Namely, the cross-inhibition is important in the base but not in the dome. In human urinary bladders, impaired contraction of the base might indicate outlet obstruction (Hirahara *et al.*, 2006). Although there are considerable species differences in the regulation of urinary bladder contraction, the results presented here raise the interesting possibility that this difference in receptor cross-inhibition might contribute to the relatively milder contraction of the base as compared to the dome in this pathological condition.

Acknowledgements

The authors are indebted for the excellent technical assistance from I. Kiss and R. Öri. This work was supported by grants from the Hungarian Scientific Research Found (OTKA F68393 and K75604), from the Hungarian Ministry of Health (ETT 186/2009) and from the Hungarian Ministry of Education (TÁMOP-4.2.2-08/2008-0019).

Conflict of interest

There are no conflicts of interest.

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Legends to figures

Fig. 1

Representative calcium transients evoked by repetitive application of ATP (180 μM) on cultured smooth muscle cells from rat urinary bladder. (A) ATP-evoked Ca^{2+} transients in urinary bladder smooth muscle cells. (B, C, D) Pooled data from 7 similar experiments (7 cells from 7 different animals) where the parameters (amplitude (B), time constant (τ) of decay (C) and maximum rate of rise (D)) of the 2nd and 3rd ATP-evoked transients were normalized to that of the first response in the given cell. None of the differences proved statistically significant ($p>0.3$, $p>0.5$ (B); $p>0.1$, $p>0.1$ (C); $p>0.5$, $p>0.3$ (D)).

Fig. 2

Representative calcium transients evoked by cholinergic and purinergic agonists in cultured and freshly isolated smooth muscle cells from adult rat urinary bladder.

The application of both ATP, carbachol(CCh)+ATP and nicotine(nic)+ATP resulted in a Ca^{2+} transient in rat smooth muscle cell from the dome (A) of the urinary bladder (ATP 180 μM, CCh 10 μM, nic 5 μM, c=16, n=52). In contrast, while carbachol prevented, nicotine did not interfere with the ATP-evoked Ca^{2+} transients in cultured (B) or freshly isolated (C) rat smooth muscle cells from the base of the urinary bladder (carbachol: c=18, n=57, nicotine: c=6, n=20 in cultures, carbachol: c=3, n=3 freshly isolated). Here, and in subsequent figures, "c" represents the number of cultures, while "n" represents the number of cells we obtained the results from. Markers show the onset of the 40s-long drug application.

Fig. 3

Pharmacological characterization of the cross-inhibition.

The muscarinic acetylcholine receptor blocker atropine (A: smooth muscle cell from the base, B: smooth muscle cell from the dome of the bladder; 1 μM) was unable to modify the purinergic-cholinergic interactions. The nicotinic acetylcholine receptor blocker α-bungarotoxin did not interfere with the ATP-evoked signals in the cells isolated from the dome (D, 500 nM), but it prevented carbachol from inhibiting the ATP-evoked calcium transients in the cells isolated from the base (C, 500 nM).

Fig. 4

Involvement of nicotinic signalling in the cross-inhibition.

The pooled data confirmed that the acetylcholine receptor agonist carbachol reduced the ATP-evoked Ca^{2+} transients in cultured rat smooth muscle cells from the base of the urinary bladder (c=18, n=57, $p<0.01$). The acetylcholine receptor blocker atropine was unable to modify the purinergic-cholinergic interactions (c=2, n=14, $p<0.05$), but the nicotinic acetylcholine receptor blocker α-bungarotoxin prevented carbachol from inhibiting the ATP-evoked calcium transients in the cells isolated from the base (c=3, n=20, $p>0.05$). Carbachol did not interfere with the ATP-evoked signals in the cells isolated from the dome (c=16, n=52, $p>0.05$), and it was not influenced by either atropine (c=2, n=10, $p>0.05$) or α-bungarotoxin (c=1, n=3, $p>0.05$). *,$^{\#}$ indicate significant difference ($^{*}p<0.01$, $^{\#}p<0.05$) between control (ATP response) and test groups.

Fig. 5

Pharmacological characterization of the purinergic pathway.

Neither α,β-methylene-ATP (abm)(A; 10 μM, n=6, c=25), nor UTP (B; 180 μM, n=5, c=29) application could induce Ca^{2+} transients in cultured smooth muscle cells of the urinary bladder. Cells were obtained from the base and from the dome of the urinary bladders.

Fig. 6

The immunostaining of nAChRα7 subunits in rat urinary bladder.

Results were obtained from cultured cells from rat urinary bladders (A), tissue sections (B) and muscle strips (C). The left column of panel A presents cultured cells from the base while the right column of panel A presents cultured cells from the dome of the urinary bladder. Green color shows smooth muscle cells stained with FITC conjugated secondary antibody. Blue color shows the nuclei of the cells stained with DAPI. Inserts in the right side of each image show negative controls. The brownish color (DAB) in the panel B presents cells which expressed nAChRα7. Left column of panel B shows section from the base, right column shows section from the dome of the bladder.

Inserts in the corners show negative controls. Scale bar represents 25 μm (A) or 250 μm (B). The Western-blot confirmed nAChRα7 protein expression in rat urinary bladder smooth muscle (C), densitometry indicated a statistically more pronounced expression in the base, than in the dome (D, $p<0.05$). The data are ratios of the signal at 56 kDa (nAChRα7 protein) over the actin signal.

Fig. 7

$P2X_2$ receptors on urinary bladder smooth muscle cell cultures.

Cultured cells from the base of the urinary bladder are presented on the left side, cultured cells from the dome are on the right. Primary antibody against $P2X_2$ receptors (1:200), secondary antibody conjugated with FITC, nuclei stained with DAPI. Scale bar indicates 25 μm, inserts show negative controls.