J. Cell. Mol. Med. Vol 13, No 9B, 2009 pp. 3069-3081

The localization of cyclo-oxygenase immuno-reactivity (COX I-IR) to the urothelium and to interstitial cells in the bladder wall

R. de Jongh [a], S. Grol [a], G. A. van Koeveringe [a], P. E. V. van Kerrebroeck [a], J. de Vente [b], J. I. Gillespie [b, c, *]

[a] Department of Urology, University Hospital Maastricht, Maastricht, The Netherlands
[b] European Graduate School of Neuroscience (EURON), The Department of Psychiatry and Neuropsychology, Maastricht University, Maastricht, The Netherlands
[c] The Uro-physiology Research Group, Institute of Cellular Medicine, The Medical School, The University, Newcastle upon Tyne, England

Received: April 8, 2008; Accepted: August 15, 2008

Abstract

Localized phasic contractions in the bladder wall (autonomous activity) have been hypothesized to be an integral part of a motor/sensory system contributing to bladder sensation. The sites responsible for generating this activity, the mechanisms involved in its propagation and modulation remain unknown. This phasic motor activity is modulated by exogenous prostaglandins. Therefore, analysis of the sites of prostaglandin production and action within the bladder wall may shed light on the mechanisms of generation and modulation of this phasic activity. In this paper we report the localization of immuno-reactivity indicative of the expression of cyclo-oxygenase enzyme type I (COX I-IR) within the bladder wall. Basically, three types of COX I-IR cell were identified: epithelial cells in the basal and intermediate layers of the urothelium, complex vimentin-positive and COX I-IR cells in the lamina propria and vimentin-negative COX I-IR cells in the lamina propria and on the surface of the inner muscle bundles. These vimentin-negative/COX I-IR cells appear to be in close apposition to a continuous network of vimentin-positive cells, which extends from the lamina propria into the inner muscle layers and subsequently into the outer muscle layers. However, the interstitial cells in this region might form a distinctly different sub-type. First, the interstitial cells in this region differ from those in the inner layer by their responsiveness to NO with a rise in cGMP. Two subtypes have been identified: cells on the surface of the muscle bundles and within the muscle bundles. Second, COX I-IR cells are not associated with the interstitial cells in the outer layers. The physiological significance for these apparent differences in the interstitial cell network is not clear. However, such differences are likely to reflect differences in the processes involved in their activation, modulation and control.

Keywords: cyclooxygenase-I • prostaglandins • interstitial cells • vimentin • autonomous bladder hypothesis

Introduction

Prostaglandins (PGs) are found in virtually all tissues where they exert a wide variety of functions including modulating smooth muscle activity, haemostasis and cytoprotection [1]. It has been known for nearly half a century that, in the bladder, PGs are released into the general circulation by the bladder in response to distension [2]. It has been established that the bladder PGs originate in both the urothelial and muscle layers [2–5]. The precise role of this endogenous PG is not known but it is well-documented that exogenous PGs alter bladder activity both *in vivo* and *in vitro* influencing voiding and smooth muscle contractility. This occurs in different species: human [6–9], rat [10–12], guinea pig [13–15], rabbit [16–19] and monkey [20, 21]. The underlying mechanisms, which involve the PGs to alter voiding patterns and induce smooth muscle contraction, are not known. Regarding PG-induced changes in voiding frequency it was envisaged that they might act directly on the afferent nerves to modulate firing and so trigger micturition at lower bladder volumes [22–24]. With regard to a direct action on the muscle, it was pointed out that they can

*Correspondence to: J.I. GILLESPIE,
The Uro-physiology Research Group, Institute of Cellular Medicine,
The Medical School, The University, Newcastle upon Tyne,
NE2 4HH, England.
Tel.: +44 191 222 6988
Fax: +44 191 222 6988
E-mail: j.i.gillespie@ncl.ac.uk

doi:10.1111/j.1582-4934.2008.00475.x

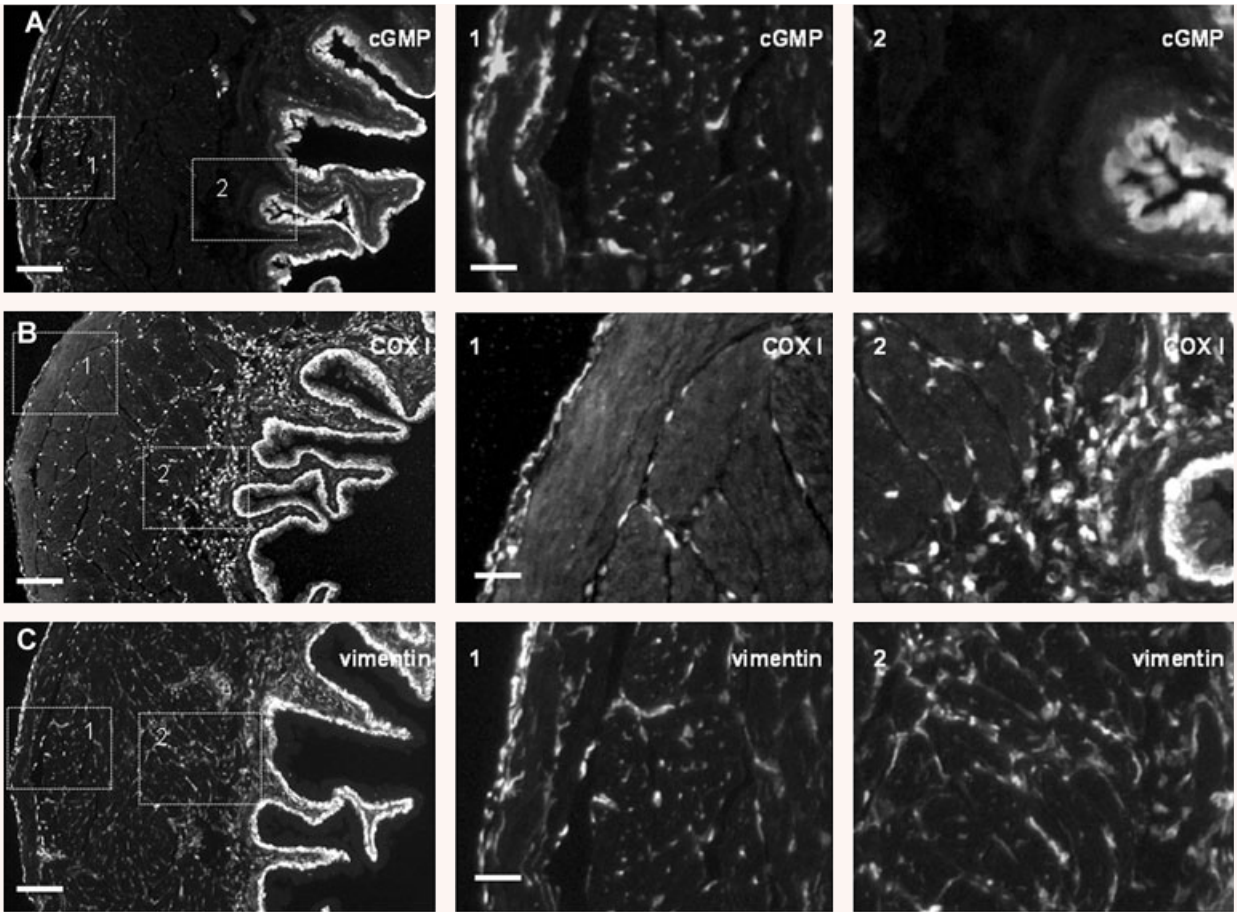

Fig. 1 The localization of COX I-IR, vimentin$^+$ cells and NO-responsive cGMP$^+$ cells in the guinea pig bladder. (**A**), (**B**) and (**C**) show, respectively, images stained with antibodies to COX I, vimentin and cGMP. In each panel, two regions are identified (1 and 2) located in the outer and inner regions of the bladder wall. For (**A**–**C**), these identified regions are shown at greater magnification in the adjacent panels. (**A**) illustrates COX I-IR. More COX I-IR cells are seen in the inner regions of the bladder. In (**B**), vimentin$^+$ cells are seen in both the outer and inner layers. In (**C**), the presence of cGMP$^+$ cells is clearly seen. No cGMP cells are seen associated with the inner muscle layer but the umbrella cells and sub-urothelial cells are cGMP$^+$. Calibrations bars: 150 μm in (**A**) and 40 μm in (**B**).

be co-released with acetylcholine at efferent nerve endings and so directly contribute to muscle excitation [20, 21]. Alternatively, they might act indirectly on pre-synaptic motor terminals to affect the release of excitatory transmitters [11, 12]. It was considered they might also inhibit acetylcholine esterase [9] or enhance myogenic bladder activity [6, 22]. In other organs, PG production in fibroblasts resulted in a decrease in collagen production [25]. It has also been reported that prostaglandin production by fibroblasts plays a role in tumour necrosis [26]. In the intestine, Powell *et al.* found that interstitial cells in the subepithelial space of the intestine are important in the organogenesis of the intestine [27], and secretion of prostaglandins by these interstitial cells is an important factor of this mechanism [27]. Therefore, it must be taken into consideration that, next to effects on contractility, PGs might have additional functions in the bladder, *e.g.* a role in cell proliferation [25].

Recently, a concept was proposed which attempts to integrate the effects of PG on the sensory elements of bladder control with its motor actions. Using the isolated bladder it was reported that exogenous PGs modulate the autonomous activity [28]. It has been argued previously that autonomous activity is part of a motor/sensory system operating within the bladder wall whereby localized contractions of the bladder wall stimulate firing in afferent nerves contributing to sensation [29, 30]. PG-induced modulation of the autonomous activity would thus increase bladder sensation and so modify voiding activity [28].

The origin of autonomous activity and how it is modulated by PGs is unknown. It has been hypothesized that it is generated within and distributed by a network of specialized cells in the bladder wall: interstitial cells [30, 31]. The precise identification and definition of what is an interstitial cell in the bladder

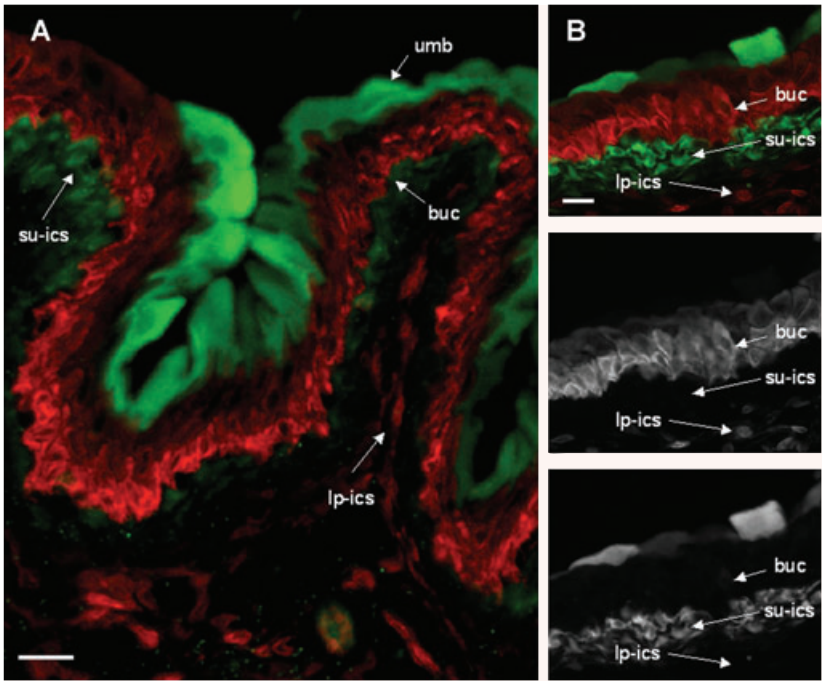

Fig. 2 cGMP- and COX I-IR associated with the urothelium in the guinea pig bladder. Panel (**A**) shows an image of the urothelium of the lateral wall stained for COX I-IR (red) and cGMP (green). The preparation was stimulated with a NO donor to elevate cGMP levels in responsive cells prior to fixation. The umbrella cells on the surface of the urothelium (umb) and cells in the sub-urothelial space, the sub-urothelial interstitial cells (su-ics) are $cGMP^{+}$. Cells in the basal regions of the urothelium (basal urothelial cells: buc) are stained intensely with the COX I antibody. In addition, COX I-IR cells are also found in the lamina propria interstitial cells (ip-ics). (**B**) illustrates a section from a different bladder processed and stained in the same way as section (**A**). The upper panel shows the combined colour image. The middle and lower panels show COX I-IR and cGMP-IR, respectively. These two panels illustrate that the $cGMP^{+}$ su-ics are negative for COX I-IR. They also show that the COX I-IR cells in the buc are separated from the COX I-IR lp-ics. Calibration bars 50 μm in (**A**) and 30 μm in (**B**).

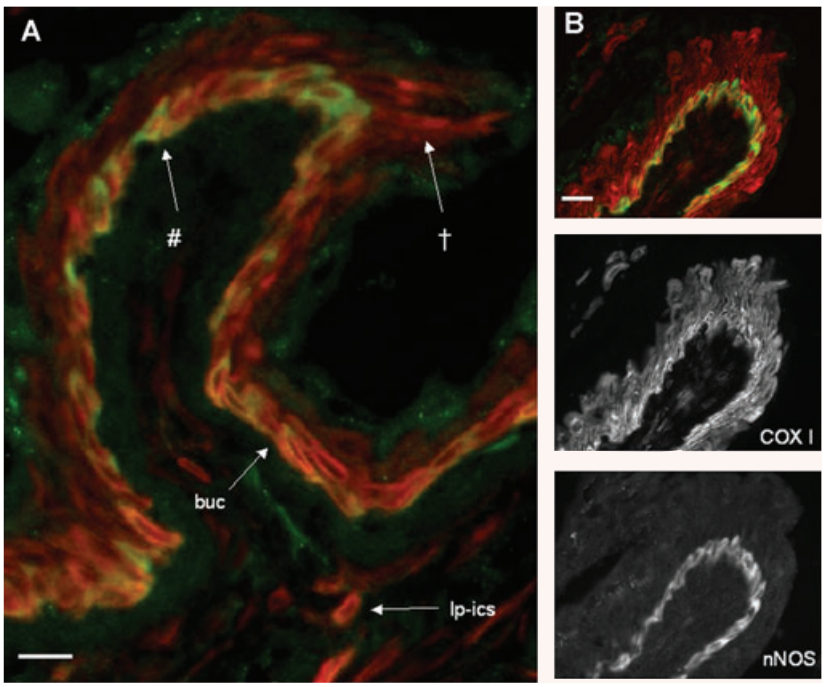

Fig. 3 The identification of cells expressing neuronal nitric oxide synthase (nNOS) and COX I-IR in the urothelial layer of the guinea pig bladder. Sections were co-stained with antibodies to nNOS (green) and COX I (red). (**A**) shows an example of the staining pattern associated with the urothelium. $nNOS^{+}$ cells (#) are seen to be located within a single layer in the basal urothelium (buc). These cells also demonstrate COX I-IR. Note that the COX I-IR extends into further cell layers within the urothelium (†). (**B**) shows a further example from a different bladder. The combined image and the individually stained images are shown. The location of nNOS to a single layer in the basal urothelium is clearly seen while the more diffuse location of the COX I-IR is apparent. Calibration bars: 30 μm in (**A**) and (**B**).

is, at present under discussion. The initial description of interstitial cells was based on their ability to show a rise in cGMP in response to nitric oxide donors [31, 32]. Different subtypes of $cGMP^{+}$ interstitial cell have been identified lying principally in the sub-urothelial layer and in the outer muscle layer [31]. Finally, the stem cell factor receptor cKit, which identifies interstitial cells in the gut, has been reported to mark cells in the bladder [33]. However, this cKit staining is proving difficult to reproduce. Direct evidence for the physiological role of interstitial cells is, so far, limited and comes from experiments

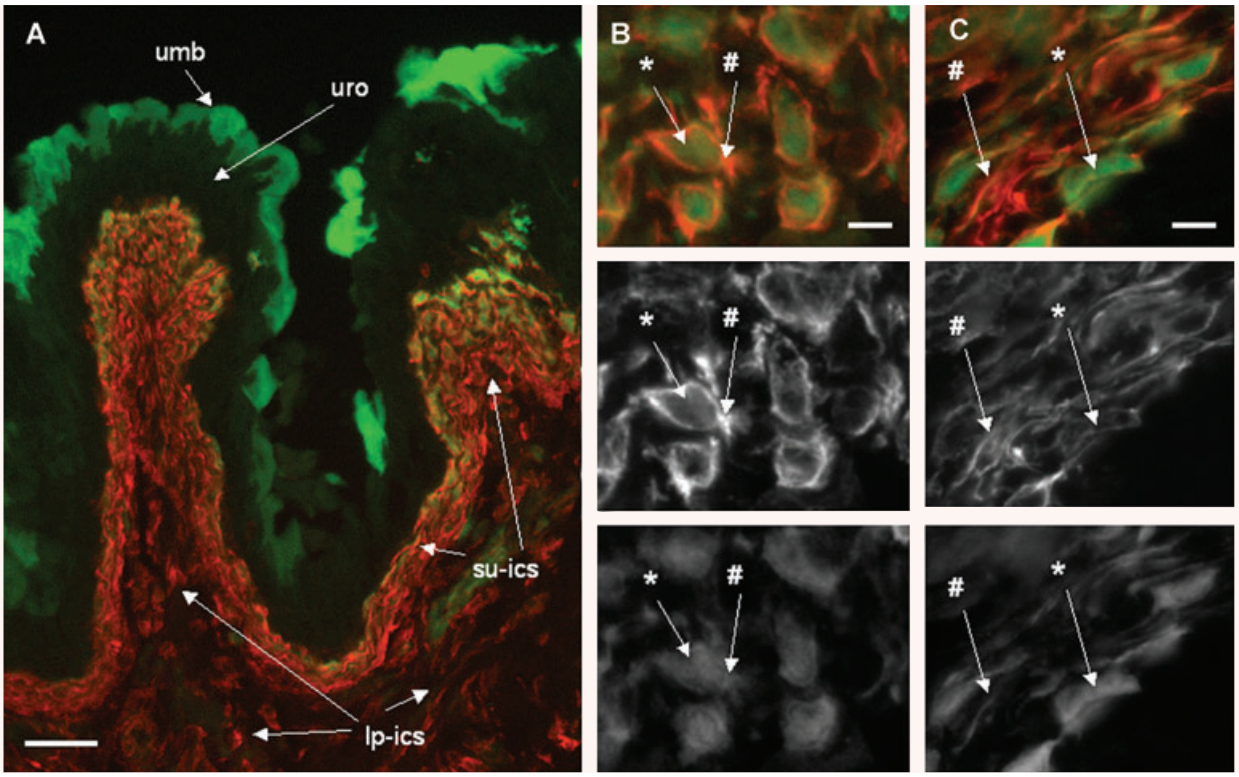

Fig. 4 The location of vimentin$^+$ structures in the urothelium and sub-urothelial space. Sections were double labelled with antibodies to show cGMP (green) and vimentin (red). Panel (**A**) shows a section from the lateral wall of the bladder illustrating the urothelium and sub-urothelial space. The umbrella cells (umb) and suburothelial interstitial cells (su-ics) are cGMP$^+$. No vimentin$^+$ structures are seen within the urothelium. Vimentin$^+$ structures are seen in the sub-urothelial space. Intense staining is seen associated with the cells immediately below the urothelium while structures laying deeper within the lamina propria are vimentin$^+$ but more defuse. (**B**) and (**C**) illustrate examples of the cells in the sub-urothelial layer at higher magnification, showing the combined colour image (upper panel: cGMP-green, vimentin-red), the cGMP image alone (middle panel) and vimentin image alone (lower panel). The cGMP$^+$ sub-urothelial interstitial cells can readily be identified (*). Vimentin staining is associated with the cell bodies of these cells being highly concentrated around the cell nuclei (#). (**C**) illustrates a further feature. Vimentin$^+$ but cGMP- fibres are seen (# in C). Calibration bars 80 μm in (**A**) and 20 μm in (**B**) and (**C**).

which show that nitric oxide (NO), which generates a rise in cGMP in the interstitial cells, also abolishes autonomous activity [34].

Since exogenous PG modulates autonomous activity [28] further insights into the origin of this activity and possible links to interstitial cells may be got from a detailed understanding of where PGs are synthesized and where they act in the bladder. PGs are synthesized by the two distinct enzymes: cyclo-oxygenase type I (COX I) and cyclo-oxygenase type II (COX II). COX I is associated with cell somata in the urothelium and with a network of cells running over the muscle bundles [28]. A detailed description of the different cell types expressing COX-I is not yet available. Therefore, the present experiments were done to characterize the cells expressing COX I in the normal bladder and to determine the relationship between these cells to the networks of interstitial cells. The distribution and characteristics of COX II expression in the bladder is the subject of a separate paper [28].

Materials and methods

Guinea pigs (7 male, weight 270–350 g) were killed by stunning and exsanguinations. The urinary bladder was removed and placed in ice-cold Kreb's solution containing 121.1 mM NaCl, 1.87 mM KCl, 1.2 mM $CaCl_2$, 1.15 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.17 mM KH_2PO_4, 11.0 mM glucose, bubbled with 5% CO_2 and 95% O_2. The bladder wall was cut into 3 or 4 sections from the base to the dome. The procedures for isolation, stimulation with the NO donor diethylamine-NONOate (DEANO Sigma-ALdrich), and detection of cGMP-immunoreactivity were as described previously [28, 31]. Preparation of the cryostat sections and immunocytochemical procedures were as described before [28, 31]. Specificity studies on the sheep or rabbit anti-formaldehyde-fixed-cGMP antisera have been published before [31] and preabsorbtion studies to ascertain the specificity of the COX I antiserum have been published before also [28]. Primary antibodies used were goat polyclonal antibody to COX I (1:2000; Santa Cruz Biotech, Santa Cruz, CA, USA), rabbit anti-nNOS antiserum (1:3000, Diasorin, Diasorin S.p.A., Saluggia, Italy), mouse anti-vimentin antiserum (1:5000; Sigma/Aldrich).

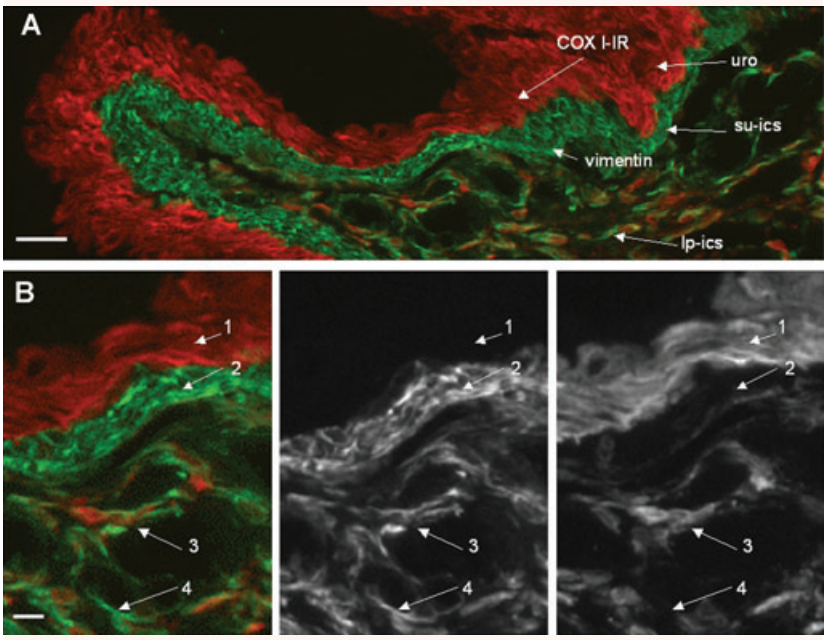

Fig. 5 Identification of COX I-IR and vimentin in the lamina propria. Sections were double labelled with antibodies to COX I (red) and vimentin (green). (**A**) shows a low power image showing the urothelium, sub-urothelial layer and the lamina propria. The COX I-IR cells are clearly visible within the urothelium (uro), the suburothelial interstitial cells (su-ics) and the lamina propria (lp-ics). Below the su-ics, there is a network of interstitial cells within the lamina propria (lp-ics). Structures positive for vimentin and with COX I-IR are seen within this layer. (**B**) shows a section of the image in (**A**) at higher magnification. The panel on the left shows the doubled labelled image (vimentin-green, COX I-red), the middle panel shows the vimentin image and the right panel shows the COX-I image. The basal urothelial layer (1), sub-urothelial layer (2) are identified. The staining pattern of the vimentin$^+$ cells in the lp is highly suggestive for a network of vimentin$^+$ structures (4). In addition, there appear to be COX I-IR cells (3).These cells are spindle shaped and are weakly positive for vimentin. They appear to lay on or between the vimentin$^+$ structures. Calibration bars 40 μm in (**A**) and 20 μm in (**B**).

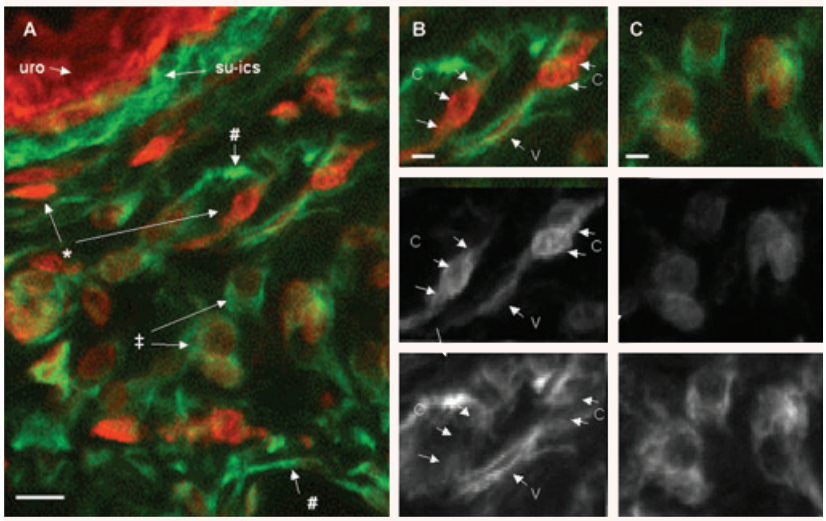

Fig. 6 Differentiation of COX I-IR and vimentin$^+$ structures in the lamina propria. (**A**) shows a region of the lateral wall. The sections were stained with antibodies to COX I (red) and vimentin (green). (**A**) shows a region of the lamina propria immediately below the urothelium. The COX I-IR of the urothelium (uro) and spindle cells in the lamina propria are seen (*) (see also Fig. 5). Also, cell processes expressing vimentin are visible. Large irregular cells are also seen which are COX I-IR and which have an extensive diffuse network of vimentin fibres (‡). (**B**) and (**C**) show selected areas of the image in (**A**) with the individual images of COX I (middle panel) and vimentin (lower panel). In (**B**), the COX I-IR cells are seen to have little or no vimentin staining. The edges of the COX I-IR cells are indicated by the arrows and (c). Vimentin$^+$ fibres are indicated (v). (**C**) illustrates the larger cells with the diffuse vimentin' network in the lamina propria. Calibration bars 20 μm in (**A**), (**B**) and (**C**).

Secondary antibodies were Alexa Fluor 488 donkey anti-sheep IgG (H+L, 1:100) and donkey anti-mouse conjugate (Molecular Probes, 1:100); Alexa Fluor 594 donkey anti-rabbit IgG conjugate (Molecular probes, 1:100); CY3 donkey anti-goat IgG conjugate (Jackson Immunoresearch, Newmarket, UK; 1:800). Sections were analysed using an Olympus AX70 fluorescence microscope, equipped with a narrow band-pass MNIBA-filter for the detection of Alexa 488, and for detection of CY3 and Alexa 594 we used a filter with a narrow excitation band, the U-M41007A filter (both from Chroma Technologies, Rockingham, VT, USA). The microscope was equipped with a cooled charge-coupled device, the Olympus digital video camera F-view. Images were stored digitally as 16 bits images by using the computer program Cell^P (Soft Imaging Systems, Olympus, Germany). The number of grey values was reduced by using a linear function to 4095. Colour images were produced by combination of the original grey values photographs using the Cell-P program. Images were arranged using the program Adobe Photoshop 7.0.1 (San Jose, CA, USA) without further processing.

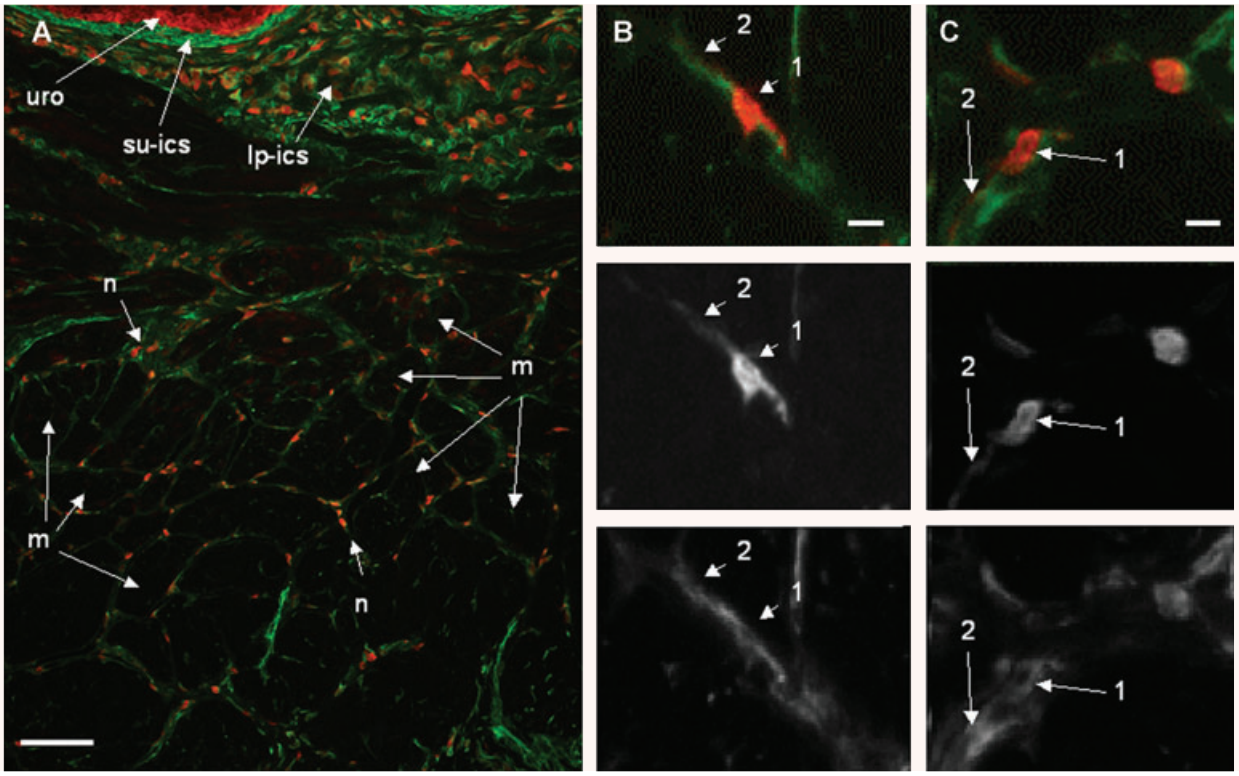

Fig. 7 A network of vimentin$^+$ processes around the muscle bundles of the inner muscle layer. Sections were double stained for COX I (red) and vimentin (green). (**A**) shows a low-power image. The COX I-IR cells in the urothelium (uro) and lamina propria (lp-ics) are seen. The vimentin$^+$ fibres of the sub-urothelial cell layer (su-ics) and within the cell network in the lamina propria are also apparent. Muscle bundles in the inner muscle layer are shown (m). (**A**) shows a network of vimentin$^+$ fibres which are associated with the muscle bundles. Collections of COX I-IR cells are seen between the muscle bundles (n). These structures are described as 'nodes'. (**B**) and (**C**) show examples of the COX I-IR cells and their relationship to the vimentin$^+$ fibres. (**B**) and (**C**) showing combined colour image (upper panel: vimentin-green, COX I-red), the COX I image alone (middle panel) and the vimentin image alone (lower panel). The COX I-IR cells (1) are weakly vimentin$^+$ and are associated with the vimentin$^+$ processes (2) of other cells. Calibration bars 240 μm in (**A**) and 10 μm in (**B**) and (**C**).

Results

The key basic observations regarding the distribution of COX I-IR in the bladder wall are illustrated in Fig. 1. All animals showed the same results. Panel A shows a low power section of the entire bladder wall. COX I-IR cells are seen within the urothelium and also associated with a network of cells, which extends from the lamina propria into the inner muscle layers. Note that there are fewer COX I-IR cells associated with the outer muscle layers. These observations are illustrated in greater detail in panels 1 and 2, which show the sub-urothelial region (1) and outer muscle layer (2) at higher magnification.

COX I-IR is therefore associated with two distinct structures: the urothelium and a network of cells in the lamina propria and around the muscle. The patter of distribution of these network cells bears a strong resemblance to the distribution of vimentin positive (vim$^+$) cells in the muscle layers bladder wall but not the distribution of NO-responsive cGMP$^+$ interstitial cells (Fig. 1B and C). Note that the cGMP$^+$ interstitial cells are found in the outer muscle layers where there is considerably less COX I-IR. These observations on COX I-IR in the urothelium and associated with the networks of interstitial cells are described in more detail below.

COX I-IR in the urothelium and sub-urothelial layer

Figure 2A illustrates a section showing the urothelium and sub-urothelial region, double stained for COX I (red) and cGMP (green). Figure 2B shows a similar section but from a different bladder illustrating the combined colour image (top) and the component COX I (middle) and cGMP (lower) images. As reported

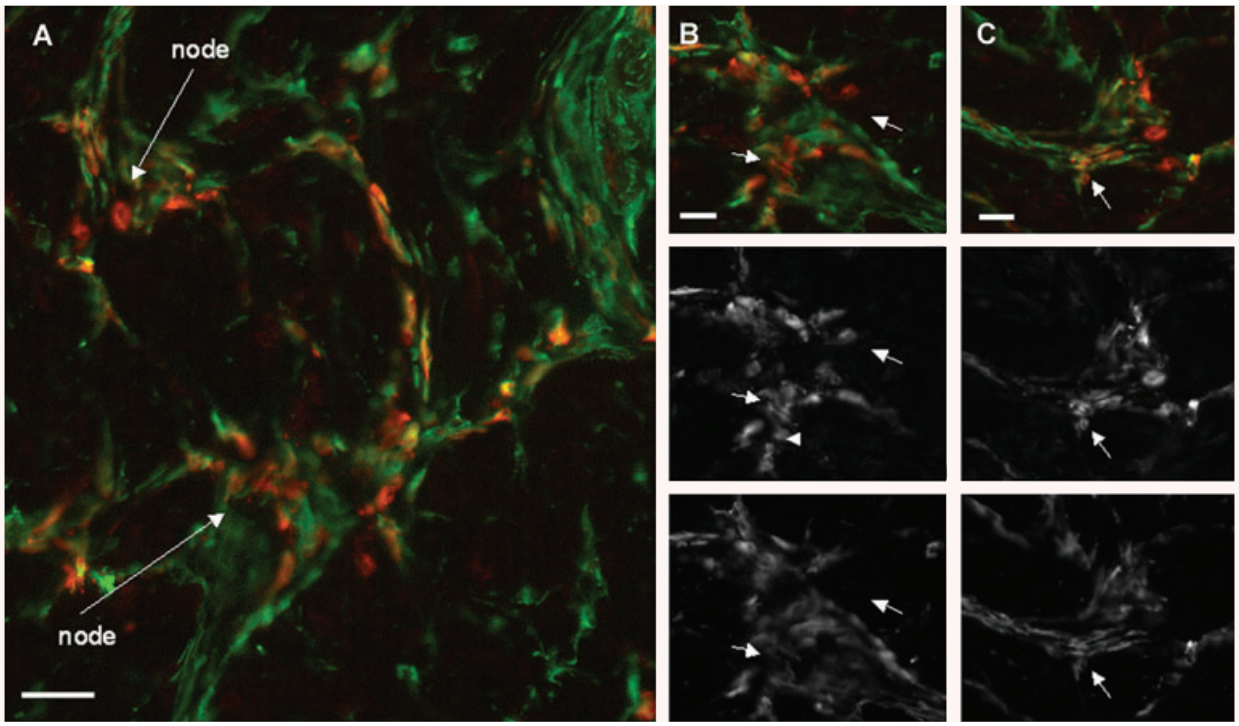

Fig. 8 Nodes associated with the interstitial cells within the lamina propria and inner muscle layer. Sections were double stained with antibodies COX I (red) and vimentin (green). (**A**) shows a region of the lamina propria. Collections of COX I-IR structures (nodes) are seen associated with collections of vimentin$^+$ fibres. (**B**) and (**C**) show selected areas of the image in (**A**) with the associated individual images to COX I (middle panel) and vimentin (lower panel). The COX I-IR structures are identified by the arrows. Little of no vimentin staining is associated with these COX I-IR structures. Calibration bars 50 μm in (**A**) and 30 μm in (**B**) and (**C**).

previously, the umbrella cells lying on the surface of the urothelium and the sub-urothelial interstitial cell layer closes to the urothelium are sensitive to exogenous NO and demonstrate a rise in cGMP (cGMP$^+$). It is also clear that COX I-IR is associated with intense staining in the intermediate and basal layers of the urothelium (buc) and weekly in cells in the lamina propria (lamina propria interstitial cells (lp-ics)). Figure 2B shows, in greater detail that, in this region of the bladder wall, the majority of the sub-urothelial cGMP$^+$ interstitial cells do not have COX I-IR.

The cells of the basal layer of the urothelium express neuronal nitric oxide synthase (nNOS) [31]. The relationship between these cells and those with COX I-IR is shown in Fig. 3. In this experiment, the sections were double stained with nNOS (green) and COX I (red). The nNOS positive (nNOS$^+$) cells are easily seen in the basal layer. These cells are also COX I-IR (showing green/yellow in the combined sections). Note that in the intermediate urothelial layer the cells are COX I-IR but not nNOS$^+$ (Fig. 3B). Thus, there are two types of COX I-IR cell in the urothelium: basal COX I-IR/nNOS$^+$ and intermediate COX I-IR/nNOS$^-$.

The layer of densely packed cells lying immediately below the urothelium, the sub-urothelial interstitial cells (su-ics), are responsive to NO with an elevation in cGMP [31]. Figure 4 illustrates a section double labelled to show these cGMP$^+$ cells (green) and the vimentin$^+$ cells (red). cGMP$^+$ umbrella cells are seen on the surface of the urothelium but there were no vim+ cells in any cell type within the urothelium. In contrast, the cells immediately below the urothelium, the su-ics, were vim$^+$ and cGMP$^+$. Vimentin staining revealed a network of intracellular fibres, whereas the cGMP staining was most intense within the cell bodies (Fig. 4B). Processes of these cells were seen to contain vim$^+$ fibres but these structures were often not strongly cGMP$^+$ indicating that there are intracellular gradients of cGMP. Vim$^+$ cells were seen in the layer of the lamina propria between the su-ic layer and the muscle layer (Fig. 4A). These cells did not demonstrate a cGMP signal and can be described as lamina propria interstitial cells (lp-ics). Thus, there appear to be different types of interstitial cell within the lamina propria: a dense region of cGMP$^+$/vim$^+$ and sparse cGMP$^-$/vim$^+$ cells.

An additional feature of the COX I/vimentin double staining becomes apparent when studying in detail the diffuse network of vim$^+$ cells in the region of the lamina propria immediately below the su-ic and above the muscle layer (Fig. 5). These cells appear

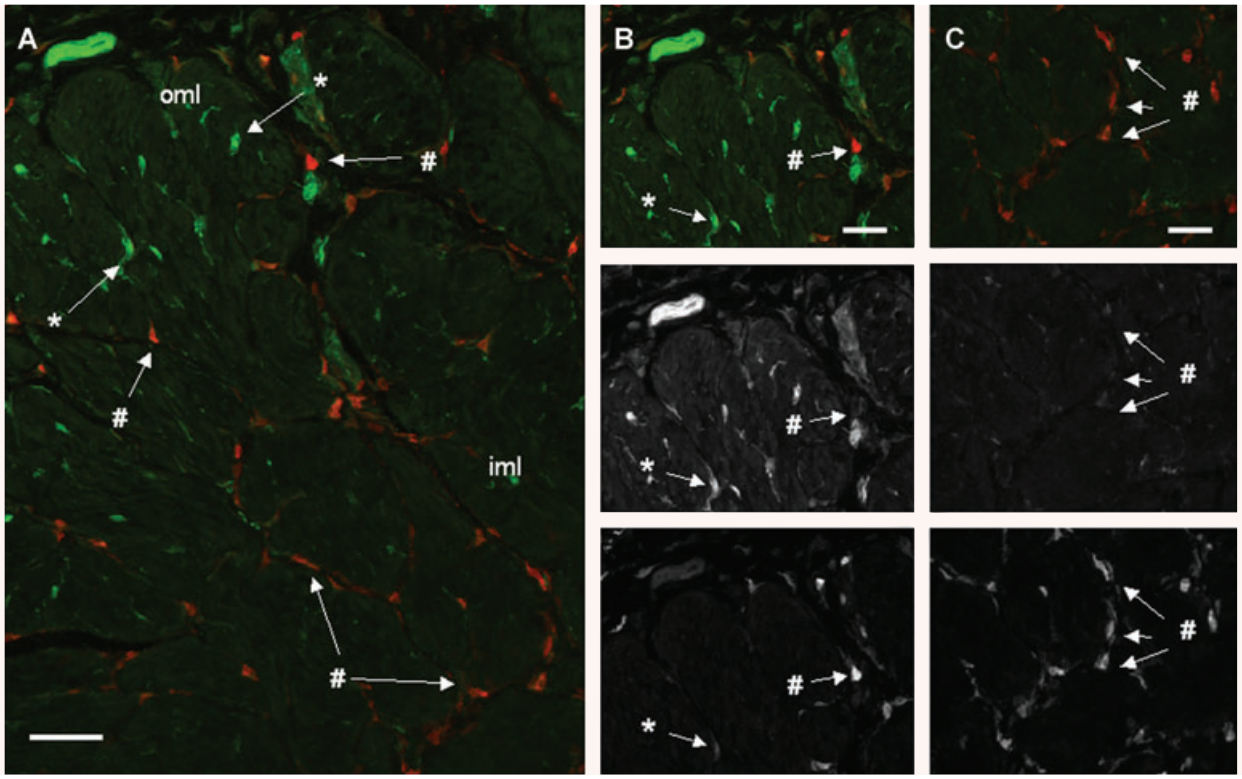

Fig. 9 The distribution of COX I-IR and cGMP$^+$ cells in the outer muscle layers. (**A**) shows an image double labelled with antibodies to cGMP (green) and COX I (red). cGMP$^+$ cells are seen in the outer muscle layer (oml) while there are no cells associated with the inner muscle layer (iml). cGMP$^+$ cells (*) and COX I-IR cells (#) are identified. (**B**) and (**C**) show regions of the image in (**A**) of the outer and inner layers respectively at higher magnification, showing the combined colour image (upper panel: cGMP-green, COX I-red), the cGMP image alone (middle panel) and COX I image alone (lower panel). In (**B**), cGMP$^+$ cells are clearly seen (*) while COX I-IR cells are scarce (#). The COX I-IR cells do not show any cGMP staining. (**C**) shows the network of COX I-IR cells in the iml and the absence of cGMP staining. Calibration bars 120 μm in (**A**) and 40 μm in (**B**) and (**C**).

to form a distinct network of inter-connecting cells contiguous with the su-ic layer and with vim$^+$ cells within the muscle layer (see below). In this section, no COX I-IR cells are visible within the su-ic layer. But, COX I-IR is seen within the cell bodies of the vim$^+$ lp-ics (Fig. 5B).

Regions of the lateral wall, particularly towards the bladder base, could be found in which the density of lp-ics was high (Figs. 6 and 7). In these regions, two different cell types were readily identified based on the expression of vimentin and COX I-IR. Figure 6A shows such a region at higher magnification. Small bipolar cells with round cell bodies, which were COX I-IR but did not stain strongly for vimentin (*) (Fig. 6B) and larger complex cells with multiple processes which showed COX I-IR and a diffuse network of vimentin fibres ((‡) and Fig. 6C), are now visible.

The network of vim$^+$ fibres was observed to continue from the lamina propria into the inner smooth muscle layer where they run primarily on the surface of the muscle bundles (Fig. 7). Here, the vim$^+$ cells appear on the surface of the muscle bundles and so can be described as surface muscle interstitial cell (sm-ics). Within this network, cell bodies were apparent, which were COX I-IR but that did not stain strongly for vimentin. Examples of such cells are shown in Fig. 7B and C. At the junction of the lamina propria and inner smooth muscle cell layer, small clusters of COX I-IR cell bodies were often observed (Fig. 8). The cell bodies in these clusters did not stain strongly for vimentin but were in close proximity to vimentin positive cell processes (Fig. 8B and C). These collections of cell bodies, which have the appearance of nodes, were also apparent, although fewer in number and with fewer cells, lying between the muscle bundles of the inner muscle layer (see Fig. 7A).

Outer muscle layers

The network of vim$^+$ sm-ics was observed to extend into the outer muscle layers of the bladder wall (Figs. 1 and 9). However, in this outer region there are few cell bodies that are COX I-IR. This points out that there must be different types of sm-ic associated

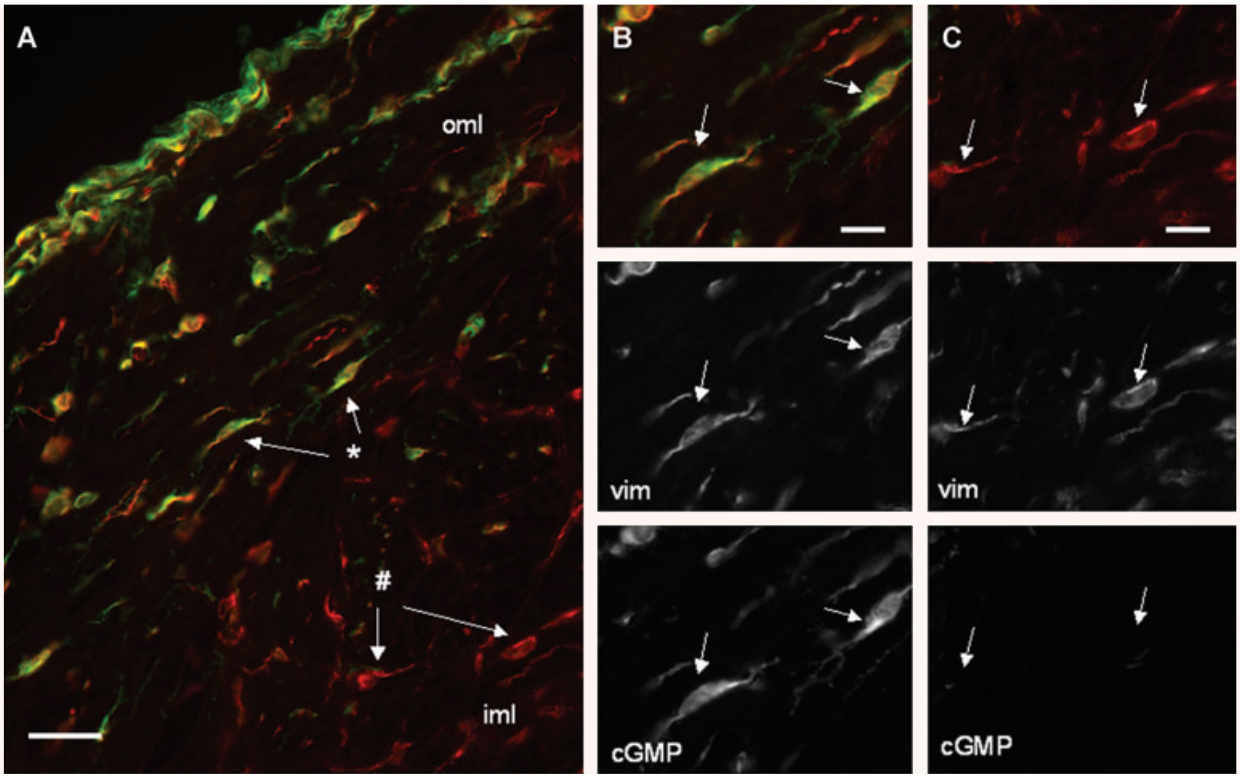

Fig. 10 Identification of interstitial cells in the muscle layers of the bladder. The sections are double labelled with antibodies to cGMP (green) and vimentin (red). (**A**) shows a low-power image identifying the outer (oml) and inner muscle layers (iml). The network of $cGMP^+$ ics associated with the outer layer and the network of $vimentin^+$ cells in the inner (*) and outer layers are readily seen. The different types of $cGMP^+$ ics are indicated by the arrows: muscle coat interstitial cells (mc-ics), surface muscle interstitial cells (sm-ics) and intra-muscular interstitial cells (im-ics). (**B**), (**C**) and (**D**) illustrate regions of the image in (**A**) at higher magnification and showing combined colour image (left panel: cGMP-green, vimentin-red), the cGMP image alone (middle panel) and vimentin image alone (left panel). (**B**) shows primarily the sm-ics. All $cGMP^+$ structures are also $vimentin^+$. (**C**) and (**D**) focus on the im-ics. $cGMP^+$ cells are seen to extend long fine processes. The cell bodies and initial parts of the cell processes are also vimentin positive. (**D**) illustrates that there is a variation on the intensity of cGMP and vimentin staining. All im-ics express vimentin. However, there are $vimentin^+$ cells which are strongly $cGMP^+$ (1) while others have little cGMP immunostaining (2 and 3). Calibration bars, 100 μm in (**A**), 40 μm in (**B**) and 20 μm in (**C**) and (**D**).

with the inner and outer muscle layers. This is supported in Fig. 10, which shows sections double labelled for cGMP (green) and vimentin (red). As has been reported previously, the sm-ics of the outer muscle layers respond to NO with a rise in cGMP [31]. The cells bodies of these outer sm-ics are clearly seen but these are not COX I-IR. In contrast, there are few $cGMP^+$ cells in the inner muscle layers (see also Fig. 1). NO-responsive cells producing cGMP are also found within the muscle bundles of the outer muscle layer: intramuscular interstitial cells (im-ics)). Figure 10 illustrates that these im-ics are also vim^+.

Thus, these data support the idea that there are different subtypes of interstitial cell associated with the lamina propria and inter-muscular spaces in the guinea pig bladder. The different cell types which have been identified and described above, based on the staining for cGMP, vimentin, COX I and nNOS, are summarized in Table 1.

Discussion

It has been known for over 30 years that PGs are released from the bladder in response to stretch, the PGs coming from both the urothelium and muscle layers [19, 24, 35, 36]. Sprem *et al.* found that intravesically administrated ketoprofen, a non-selective COX inhibitor, reduced detrusor instability [37]. Based on these findings, it has been argued that the PGs play central roles not only in bladder physiology but also in the generation of bladder patho-physiology [2–5, 38–40]. This led to the trial of cyclooxygenase inhibitors for the treatment of bladder over-activity [37, 41]. One indication as to the possible role of PGs has come from experiments involving the infusion of PG into the bladder lumen. When this is done, it gives rise to an increase in micturition frequency and the incidence of non-voiding phasic contractions between voiding episodes [42, 43]. Prostaglandin production

Table 1 Location of cGMP, vimentin, COX I and nNOS in the bladder

Cell location	Cell type	Staining			
		cGMP	Vimentin	COX I	nNOS
Urothelium					
	Umbrella	+	−	−	−
	Intermediate	−	−	+	−
	Basal	−	−	+	+
Lamina propria					
	Superficial su-ics	+	+	−	−
	Deep su-ics	−	+	−	−
	Lp-ics #1	−	+	−	−
	lp-ics #2	−	−	+	−
	lp-ics #3	−	+	+	−
Inner muscle					
	sm-ics #1	−	+	−	−
	sm-ics #2	−	−	+	−
	im-ics	−	+	−	−
Outer muscle					
	sm-ics	+	+	−	−
	im-ics	+	+	−	−
Muscle coat					
	mc-ics	+	+	−	−

ip-ics : lamina propria interstitial cells
- lp-ics#1: lamina propria interstitial cells type #1 (only vimentin positive lp-ics)
- ip-ics#2: lamina propria interstitial cells type #2 (only COX I positive lp-ics)
- lp-ics#3: lamina propria interstitial cells type #3 (lp-ics that are both vimentin and COX I positive)
- sm-ics: surface muscle interstitial cells
- sm-ics#1: surface muscle interstitial cells type #1 (vimentin positive sm-ics)
- sm-ics#2: surface muscle interstitial cells type #2 (COX I positive sm-ics)
- im-ics: intramuscular interstitial cells
- mc-ics: muscle coat interstitial cells

in the bladder serves also other functions. Bachteeva *et al.* reported that PGs play a role in the osmotic water permeability of the frog urinary bladder [44]. Several subtypes of prostaglandin receptors have been found in the bladder. It has been reported that the urothelium contains both the prostaglandin receptor subtype E2 (EP2), which plays a role in osmoregulation [45], as well as the EP1 receptor, which plays a role in the micturation reflex [46]. Schröder *et al.* reported that the EP1 receptor has a role in the development of detrusor overactivity caused by PGE2 and outlet obstruction [47]. The details of the mechanisms underlying this action of PGs on voiding frequency are not known.

Several possible mechanisms have been put forward. One idea is that the PGs have a direct effect on bladder afferent nerve fibres [48]. By increasing afferent nerve activity, this would result in a more frequent activation of the micturition reflex. It is well-documented that PGs cause a sensitization of cutaneous nociceptors [49]. This might also occur in the bladder. Support for the idea comes indirectly from experiments in which the bladder was treated with capsaicin to remove the afferent contribution of C fibres. After functionally removing the C fibres, the PG-induced increase in micturition frequency of micturition was reduced [40]. A different mechanism involving an indirect action of PGs on afferent nerves has recently been proposed. It is known that small localized contractions occur in the bladder wall of many species. This complex activity is hypothesized to be the motor component of a motor/sensory system involved in the generation of afferent firing and bladder sensation [29, 31]. Using the isolated guinea pig bladder it has been shown that PGs increase the frequency of this phasic motor activity [28]. Thus, the increased phasic motor activity could result in an increase in afferent discharges and in so doing influence the point at which voiding is triggered [28].

As discussed above, PGs are synthesized within the lamina propria and muscle layers. The present observations extend this broad observation and demonstrate specifically that the expression of COX I-IR predominates within two general cell systems in the bladder wall: (i) cells within the basal and intermediate layers of the urothelium and (ii) within a population of small cells that are closely associated with a network of vimentin positive cells. These vimentin positive cells are present through out the sub-urothelial space of the lamina propria and extend over the surface of the muscle bundles, which make up the inner layers. It is interesting and important to note that no COX I-IR was seen within the muscles indicating that, in the guinea pig, it is not the smooth muscle that is producing PG but the cells associated with the vimentin network.

These observations raise intriguing questions regarding the mechanisms of action of PGs in the guinea pig bladder. One hypothesis might be that the COX I in the basal urothelial cell layers is activated by bladder distension. The PGs produced here could diffuse the relatively short distance to the sub-urothelial space in which sensory afferent nerve fibres are found. Thus, this arrangement would represent the site where there is a PG-induced direct modulation of afferent nerves.

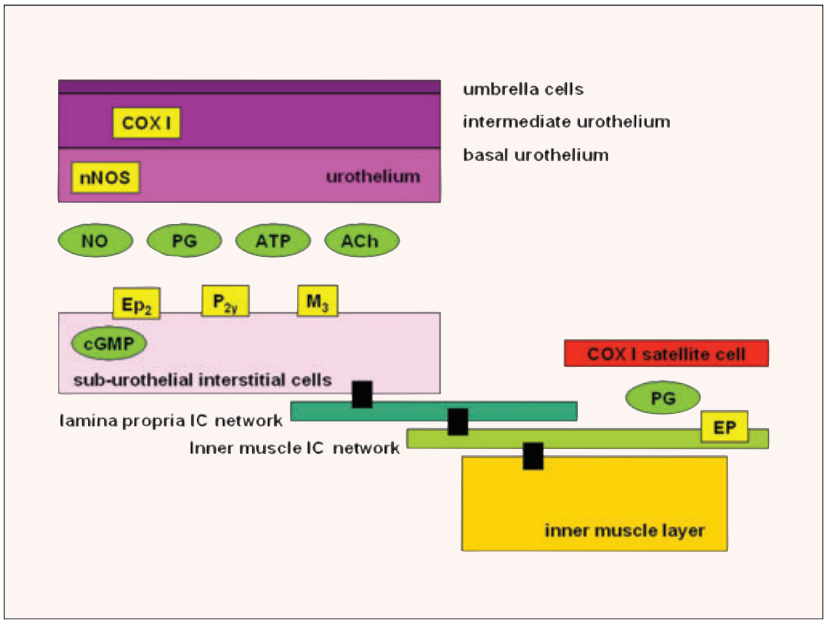

Fig. 11 Summary diagram illustrating the arrangement of cells found in the wall of the guinea pig bladder and their hypothesized interaction. The urothelium releases NO, PG, ATP and acetylcholine in response to stretch. The production of NO and PG in the urothelium is interrelated. The substances released act on afferent nerves but may also act upon the sub-urothelial interstitial cells. This network is in direct contact with a network of vimentin positive interstitial cells which run in the sub-urothelial space of the lamina propria and extend over the surface of the inner muscle bundles. The mechanisms connecting cells in this network can be via gap junctions (black squares). COX I-IR cells (satellite cells) are associated with the vimentin^{+} interstitial cell network where they influence the activity of the network.

However, the abundance of COX I-IR in the urothelium of the lateral wall and the relative paucity of afferent nerves indicate that this is not the only role for PGs produced by the urothelium in the lateral wall.

The present data also show that the COX I-IR cells in the base of the urothelium also express nNOS. Like PG, NO is known to be produced by the urothelium in response to stretch [50]. It is also known, on other cell systems, that PG production is influenced by NO and, conversely, that NO production is influenced by PGs [51, 52]. Other signals also originate in the urothelium in response to stretch. Specifically, ATP has been shown to be released [53] and one of its actions is to influence afferent nerve firing [54]. Also, there are reports that acetylcholine is released from the urothelium [55]. Thus, we conclude that there is a complex and inter-related release of signalling substances from the urothelium in response to mechanical deformation.

To add to this complexity, the cell layer immediately below the urothelium, the sub-urothelial interstitial cell (su-ic) layer, can be a possible location for further integration of urothelial derived signals. These su-ics, in the guinea pig and human, respond to both exogenous and endogenous NO demonstrating a rise in cGMP [30, 32]. These cells are also immuno-reactive for antibodies to the type 3 muscarinic receptor (M_3) (unpublished observation), purinergic receptor [56] and the type 2 prostaglandin receptor (EP2) (unpublished observation). Thus, the integrated output of PG, NO, ATP and cholinergic stimuli from the urothelium can be further integrated and modulated on the su-ics. The specific function of the su-ics cells is, at present, not known.

Indeed, there is indirect support for such a possible integration on the sub-urothelial cells. The su-ics are in close relation with the network of interstitial cells of the lamina propria and on the surface of the inner muscles. The autonomous bladder hypothesis suggests that, based on the observations of Lagou *et al.* [34] that the muscle interstitial cells are involved in the activation and co-ordination of complex phasic activity (autonomous activity) within the smooth muscle [57] and it is this activity that is the motor component of the motor sensory system [30]. The amplitude and frequency of the autonomous activity are increased by cholinergic agonists [58, 59] and ATP [60]. If this type of activity is generated within a network of interstital cells this points out that there are M_3/ATP-activated pacemakers linked to a distributed network [59].

It has also been shown that bladder distension alters autonomous activity: an increase in bladder volume increases autonomous activity, whereas a decrease in bladder volume inhibits it [61]. The complex scheme outlined above might provide an explanation for these volume-related events. Mechanical deformation of the urothelium in the lateral wall triggers a complex cascade of interacting signals within the urothelial epithelium resulting in the release of signals into the sub-urothelial space. There, these urothelial signals are further integrated on the su-ics. Activity in the su-ics is then distributed to the muscle via the vimentin positive interstitial cell network resulting in the excitatory and inhibitory effects on the phasic activity (see Fig. 11).

Prostaglandin synthesis has been associated with the smooth muscle [24]. The precise cell types and their location have not been considered in detail; it was simply assumed that it originated in smooth muscle cells. The present observations put forward a new idea that, at least in the guinea pig, it is the COX I-IR cells associated with the network of vimentin positive interstitial cells on the surface of the muscle bundles that are

responsible for synthesizing PG in this layer. As already discussed, one of the possible roles of this network of muscle interstitial cells is to generate and distribute signals leading to phasic contractions in the bladder wall [34, 57, 62]. Thus, the micro-anatomical arrangement of cells in the muscle layer appears to involve COX I-IR (PG producing) cells contacting vimentin interstitial cells in close apposition to smooth muscle. Functionally, it can be hypothesized that this arrangement involves a PG regulation of activity in the vimentin network and consequently an input to the muscle. The actions of PG on the muscle would thus be indirect being interceded by the network of interstitial cells (see Fig. 11).

The present observations also demonstrate a difference in the interstitial cells in the outer and inner muscle layers of the bladder wall, specifically the reduction in number of COX I-IR cells in the outer layers. Differences have been noted before [31, 34]. However, the functional roles for these apparently different types of muscle interstitial cell are not known. What is clear is that the network of interstitial cells is complex and may sub-serve several different functions.

In conclusion, it is now quite clear that there are several complex signalling systems operating within the bladder wall. In addition, it is clear that these signals act within cell-systems that interact between the urothelium, interstitial cells, muscle, sensory fibres and intra-mural ganglia. It is a major challenge for the future to unravel this complexity not only in relation to the physiology and pharmacology of the bladder but also in relation to the origins and treatment of bladder pathology.

References

1. **Khan MA, Thompson CS, Mumtaz FH, *et al*.** Role of prostaglandins in the urinary bladder: an update. *Prostaglandins Leukot Essent Fatty Acids.* 1998; 59: 415–22.
2. **Gilmore NJ, Vane JR.** Hormones released into the circulation when the urinary bladder of the anaesthetized dog is distended. *Clin Sci.* 1971; 41: 69–83.
3. **Abrams PH, Sykes JA, Rose AJ, *et al*.** The synthesis and release of prostaglandins by human urinary bladder muscle *in vitro*. *Invest Urol.* 1979; 16: 346–8.
4. **Jeremy JY, Tsang V, Mikhailidis DP, *et al*.** Eicosanoid synthesis by human urinary bladder mucosa: pathological implications. *Br J Urol.* 1987; 59: 36–9.
5. **Mikhailidis DP, Jeremy JY, Dandona P.** Urinary bladder prostanoids – their synthesis, function and possible role in the pathogenesis and treatment of disease. *J Urol.* 1987; 137: 577–82.
6. **Bultitude MI, Hills NH, Shuttleworth KE.** Clinical and experimental studies on the action of prostaglandins and their synthesis inhibitors on detrusor muscle *in vitro* and *in vivo*. *Br J Urol.* 1976; 48: 631–7.
7. **Hills NH.** Prostaglandins and tone in isolated strips of mammalian bladder. *Br J Pharmacol.* 1976; 57: 464P–5P.
8. **Andersson KE, Ek A, Persson CG.** Effects of prostaglandins on the isolated human bladder and urethra. *Acta Physiol Scand.* 1977; 100: 165–71.
9. **Borda E, Contreras-Ortiz N, Gutnisky R, *et al*.** *In vitro* effect of acetylcholine and bethanechol on the contractions of the human detrusor muscle. Influence of prostaglandins. *Arch Int Pharmacodyn Ther.* 1982; 259: 31–9.
10. **Brown C, Burnstock G, Cocks T.** Effects of adenosine 5'-triphosphate (ATP) and beta-gamma-methylene ATP on the rat urinary bladder. *Br J Pharmacol.* 1979; 65: 97–102.
11. **Choo LK, Mitchelson F.** The role of prostaglandins in the excitatory innervation of the rat urinary bladder. *Prostaglandins.* 1977; 13: 917–26.
12. **Choo LK, Mitchelson F.** The effect of indomethacin and adenosine 5'- triphosphate on the excitatory innervation of the rate urinary bladder. *Can J Physiol Pharmacol.* 1980; 58: 1042–8.
13. **Burnstock G, Cocks T, Crowe R, *et al*.** Purinergic innervation of the guinea-pig urinary bladder. *Br J Pharmacol.* 1978; 63: 125–38.
14. **Burnstock G, Cocks T, Kasakov L, *et al*.** Direct evidence for ATP release from non-adrenergic, non-cholinergic ("purinergic") nerves in the guinea-pig taenia coli and bladder. *Eur J Pharmacol.* 1978; 1549: 145–9.
15. **Johns A.** The effect of indomethacin and substance P on the guinea pig urinary bladder. *Life Sci.* 1981; 2629: 1803–9.
16. **Dean DM, Downie JW.** Interaction of prostaglandins and adenosine 5'-triphosphate in the noncholinergic neurotransmission in rabbit detrusor. *Prostaglandins.* 1978; 16: 245–51.
17. **Husted S, Sjogren C, KE AN.** Role of prostaglandins in the responses of rabbit detrusor to non-cholinergic, non-adrenergic nerve stimulation and to ATP. *Arch Int Pharmacodyn Ther.* 1980; 246: 84–97.
18. **Andersson KE, Husted S, Sjogren C.** Contribution of prostaglandins to the adenosine triphosphate-induced contraction of rabbit urinary bladder. *Br J Pharmacol.* 1980; 70: 443–52.
19. **Downie JW, Larsson C.** Prostaglandin involvement in contractions evoked in rabbit detrusor by field stimulation and by adenosine 5'-triphosphate. *Can J Physiol Pharmacol.* 1981; 59: 253–60.
20. **Johns A, Paton DM.** Evidence for a role of prostaglandins in atropineresistant transmission in the mammalian urinary bladder. *Prostaglandins.* 1976; 11: 595–7.
21. **Johns A, Paton DM.** Effect of indomethacin on atropine-resistant transmission in rabbit and monkey urinary bladder: evidence for involvement of prostaglandins in transmission. *Prostaglandins.* 1977; 13: 245–54.
22. **Andersson KE, Sjogren C.** Aspects on the physiology and pharmacology of the bladder and urethra. *Prog Neurobiol.* 1982; 19: 71–89.
23. **Andersson KE.** Current concepts in the treatment of disorders of micturition. *Drugs.* 1988; 35: 477–94.
24. **Anderson KE.** Pharmacology of lower urinary tract smooth muscles and penile erectile tissues. *Pharmacol Rev.* 1993; 45: 253–308.
25. **Goldstein RH, Polgar P.** The effect and interaction of bradykinin and prostaglandins on protein and collagen production by lung fibroblasts. *J Biol Chem.* 1982; 10257: 8630–3.
26. **Elias JA.** Tumor necrosis factor interacts with interleukin-1 and interferons to inhibit fibroblast proliferation *via* fibroblast prostaglandin-dependent and -independent mechanisms. *Am Rev Respir Dis.* 1988; 138: 652–8.
27. **Powell DW, Mifflin RC, Valentich JD, *et al*.** Myofibroblasts. II. Intestinal subepithelial

myofibroblasts. *Am J Physiol*. 1999; 277: 183–201.

28. **de Jongh R, van Koeveringe GA, van Kerrebroeck PE, *et al*.** The effects of exogenous prostaglandins and the identification of constitutive cyclooxygenase I and II immunoreactivity in the normal guinea pig bladder. *BJU Int*. 2007; 100: 419–29.
29. **Starling E.** Elements of human physiology. 8th ed. Churchhill: London; 1907.
30. **Gillespie JI.** The autonomous bladder: a view of the origin of bladder overactivity and sensory urge. *BJU Int*. 2004; 93: 478–83.
31. **Gillespie JI, Markerink-van Ittersum M, de Vente J.** cGMP-generating cells in the bladder wall: identification of distinct networks of interstitial cells. *BJU Int*. 2004; 94: 1114–24.
32. **Smet PJ, Jonavicius J, Marshall VR, *et al*.** Distribution of nitric oxide synthase-immunoreactive nerves and identification of the cellular targets of nitric oxide in guinea-pig and human urinary bladder by cGMP immunohistochemistry. *Neuroscience*. 1996; 71: 337–48.
33. **McCloskey KD, Gurney AM.** Kit positive cells in the guinea pig bladder. *J Urol*. 2002; 168: 832–6.
34. **Lagou M, Drake MJ, Markerink VANIM, *et al*.** Interstitial cells and phasic activity in the isolated mouse bladder. *BJU Int*. 2006; 98: 643–50.
35. **Brown WW, Zenser TV, Davis BB.** Prostaglandin E2 production by rabbit urinary bladder. *Am J Physiol*. 1980; 239: 452–8.
36. **Kasakov LN, Vlaskovska MV.** Profile of prostaglandins generated in the detrusor muscle of rat urinary bladder: effects of adenosine triphosphate and adenosine. *Eur J Pharmacol*. 1985; 31113: 431–6.
37. **Sprem M, Milicic D, Oreskovic S, *et al*.** Intravesically administered ketoprofen in treatment of detrusor instability: cross-over study. *Croat Med J*. 2000; 41: 423–7.
38. **Maggi CA.** Prostanoids as local modulators of reflex micturition. *Pharmacol Res*. 1992; 25: 13–20.
39. **Maggi CA, Evangelista S, Grimaldi G, *et al*.** Evidence for the involvement of arachidonic acid metabolites in spontaneous and drug-induced contractions of rat urinary bladder. *J Pharmacol Exp Ther*. 1984; 230: 500–13.
40. **Maggi CA, Giuliani S, Conte B, *et al*.** Prostanoids modulate reflex micturition by acting through capsaicin-sensitive afferents. *Eur J Pharmacol*. 1988; 12145: 105–12.
41. **Angelico P, Guarneri L, Velasco C, *et al*.** Effect of cyclooxygenase inhibitors on the micturition reflex in rats: correlation with inhibition of cyclooxygenase isozymes. *BJU Int*. 2006; 97: 837–46.
42. **Ishizuka O, Mattiasson A, Andersson KE.** Prostaglandin E2-induced bladder hyperactivity in normal, conscious rats: involvement of tachykinins? *J Urol*. 1995; 153: 2034–8.
43. **Takeda H, Yamazaki Y, Igawa Y, *et al*.** Effects of beta(3)-adrenoceptor stimulation on prostaglandin E(2)-induced bladder hyperactivity and on the cardiovascular system in conscious rats. *Neurourol Urodyn*. 2002; 21: 558–65.
44. **Bachteeva V, Fock E, Lavrova E, *et al*.** Prostaglandin E2 inhibits vasotocin-induced osmotic water permeability in the frog urinary bladder by EP1-receptor-mediated activation of NO/cGMP pathway. *Am J Physiol Regul Integr Comp Physiol*. 2007; 293: 528–37.
45. **Parnova RG, Bakhteeva VT, Lavrova EA.** Various effects of prostaglandin E2 on reabsorption of water and urea in the amphibia osmosis-regulating epithelium. *Ross Fiziol Zh Im I M Sechenova*. 2001; 87: 1662–72.
46. **Wang X, Momota Y, Yanase H, *et al*.** Urothelium EP1 receptor facilitates the micturition reflex in mice. *Biomed Res*. 2008; 29: 105–11.
47. **Schroder A, Newgreen D, Andersson KE.** Detrusor responses to prostaglandin E2 and bladder outlet obstruction in wild-type and Ep1 receptor knockout mice. *J Urol*. 2004; 172: 1166–70.
48. **Andersson KE, Arner A.** Urinary bladder contraction and relaxation: physiology and pathophysiology. *Physiol Rev*. 2004; 84: 935–86.
49. **Cohen RH, Perl ER.** Chemical factors in the sensitization of cutaneous nociceptors. *Prog Brain Res*. 1988; 74: 201–6.
50. **Birder LA, Apodaca G, De Groat WC, *et al*.** Adrenergic- and capsaicin-evoked nitric oxide release from urothelium and afferent nerves in urinary bladder. *Am J Physiol*. 1998; 275: 226–9.
51. **Salvemini D, Misko TP, Masferrer JL, *et al*.** Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci USA*. 1993; 190: 7240–4.
52. **Salvemini D, Seibert K, Masferrer JL, *et al*.** Endogenous nitric oxide enhances prostaglandin production in a model of renal inflammation. *J Clin Invest*. 1994; 93: 1940–7.
53. **Ferguson DR, Kennedy I, Burton TJ.** ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes–a possible sensory mechanism? *J Physiol*. 1997; 1505: 503–11.
54. **Lee HY, Bardini M, Burnstock G.** Distribution of P2X receptors in the urinary bladder and the ureter of the rat. *J Urol*. 2000; 163: 2002–7.
55. **Yoshida M, Miyamae K, Iwashita H, *et al*.** Management of detrusor dysfunction in the elderly: changes in acetylcholine and adenosine triphosphate release during aging. *Urology*. 2004; 63: 17–23.
56. **Fry CH, Ikeda Y, Harvey R, *et al*.** Control of bladder function by peripheral nerves: avenues for novel drug targets. *Urology*. 2004; 63: 24–31.
57. **Hashitani H, Yanai Y, Suzuki H.** Role of interstitial cells and gap junctions in the transmission of spontaneous Ca2+ signals in detrusor smooth muscles of the guinea-pig urinary bladder. *J Physiol*. 2004; 1559: 567–81.
58. **Drake MJ, Harvey IJ, Gillespie JI.** Autonomous activity in the isolated guinea pig bladder. *Exp Physiol*. 2003; 88: 19–30.
59. **Finney SM, Stewart LH, Gillespie JI.** Cholinergic activation of phasic activity in the isolated bladder: possible evidence for M3- and M2-dependent components of a motor/sensory system. *BJU Int*. 2007; 100: 668–78.
60. **Gillespie JI.** Modulation of autonomous contractile activity in the isolated whole bladder of the guinea pig. *BJU Int*. 2004; 93: 393–400.
61. **Lagou M, Drake MJ, Gillespie JI.** Volume-induced effects on the isolated bladder: a possible local reflex. *BJU Int*. 2004; 94: 1356–65.
62. **Gillespie JI, Harvey IJ, Drake MJ.** Agonist- and nerve-induced phasic activity in the isolated whole bladder of the guinea pig: evidence for two types of bladder activity. *Exp physiol*. 2003; 88: 343–57.