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Adrenergic signaling elements in the bladder wall of the adult rat

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ABSTRACT

A growing body of work is describing the absence of a significant sympathetic innervation of the detrusor implying little sympathetic regulation of bladder contractility. However, low doses of adrenergic agonists are capable of relaxing the bladder smooth muscle. If these effects underpin a physiological response then the cellular nature and operation of this system are currently unknown.

The present immunohistochemistry study was done to explore the existence of alternative adrenergic signaling elements in the rat bladder wall. Using antibodies to tyrosine hydroxylase (TH) and vesicular mono-amine transporter (vmat), few adrenergic nerves were found in the detrusor although TH immunoreactive (IR) nerves were apparent in the bladder neck. TH-IR and vmat-IR nerves were however abundant surrounding blood vessels. A population of vmat-IR cells was found within the network of interstitial cells that surround the detrusor muscle bundles. These vmat-IR cells were not or only weakly TH-IR. This suggests that these interstitial cells have the capacity to store and release catecholamines that may involve noradrenaline. Cells expressing the β_1-adrenoceptor (β_1AR-IR) were also detected within the interstitial cell network. Double staining with antibodies to β_1AR and vmat suggests that the majority of vmat-IR interstitial cells show β_1AR-IR indicative of an autocrine signaling system.

In conclusion, a population of interstitial cells has the machinery to store, release and respond to catecholamines. Thus, there might exist a non-neuronal β-adrenergic system operating in the bladder wall possibly linked to one component of motor activity, micro-contractions, a system that may be involved in mechanisms underpinning bladder sensation.

1. Introduction

It has been reported that the smooth muscle of the bladder base contains α-adrenoceptors facilitating bladder outlet contraction while β-adrenoceptors are found in the lateral wall, linked to bladder relaxation (Andersson and Arner, 2004; Levin and Wein, 1979). mRNA for all three β-adrenoceptor isoforms (β_1, β_2, β_3) has been identified in the detrusor (Fujimura et al., 1999; Seguchi et al., 1998). Using selective β-adrenoceptor agonists, it was proposed that the β_3-adrenoceptor isoform was the most relevant in human detrusor relaxation (Igawa et al., 1998; Svalo et al., 2013). These conclusions led to the idea that the β_3-adrenoceptor system would be a potential therapeutic target for the management of bladder pathology: overactive bladder (OAB) (Fujimura et al., 1999; Sacco and Bientinesi, 2012). Indeed, drugs such as mirabegron have been shown to reduce symptoms of urge and frequency and to improve quality of life scores in patients with OAB (Sacco and Bientinesi, 2012).

Implicit in this concept is the idea that the β-adrenoceptors in the detrusor would be activated by adrenergic nerves of the sympathetic system: sympathetic activity promoting relaxation and enhancing bladder capacity. However, it has been demonstrated that there is only a sparse adrenergic innervation of the detrusor in both rat and human (Gosling et al., 1999; Watanabe and Yamamoto, 1979). Thus, the nature of any physiological activation of β-adrenoceptors in the bladder wall is not fully understood.

Recent observations have led to the suggestion that the organization of the contractile elements of the bladder wall might be more complex than previously thought (Gillespie, 2004; Vahabi and Drake, 2015). In vitro data suggests that rather than being inactive until stimulated, the smooth muscle in the bladder wall is involved in generating complex intrinsic micro-contractile activity (Gillespie et al., 2015a). Such activity influences the compliance of the bladder wall, but it is also thought to be related to complex non-voiding contractile activity seen in vivo, that may be part of a motor-sensory system involved in the detection of bladder volume (Gillespie, 2004). The micro-contractile activity may be generated within the smooth muscle cells themselves but it is

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now likely that a network of interstitial cells is involved in the generation and modulation of this complex activity (Gillespie et al., 2015a; Gillespie et al., 2015c; Hashitani, 2006; Hashitani et al., 2004; Persyn et al., 2016). In isolated rat bladder strips, it has been reported that isoprenaline reduces this intrinsic micro-contractile activity (Gillespie et al., 2015a; Gillespie et al., 2015b). Using selective adrenergic receptor antagonists, it has been suggested that this inhibition is primarily via a β_1-adrenoceptor mechanism (Gillespie et al., 2015b).

To further understand the physiological responses found in previous functional studies, the present study was done to identify and characterize adrenergic nerves and signaling elements in the bladder wall of the rat using immunohistochemistry. This structural approach combined with the previous functional data reinforces the concept that the detrusor is controlled by complex systems influencing different physiological systems operating within the bladder wall.

2. Materials and methods

2.1. Tissue isolation and preparation

The isolation of tissue and preparation of sections has been described in previous publications (Gillespie et al., 2006). Briefly, Wistar rats (female, n = 11 weight 150–220 g) were killed by stunning and cervical dislocation (Approved UK Home office: scheduled 1 killing). The bladders were surgically removed and immersed immediately in 4% paraformaldehyde in phosphate buffer saline (PBS) for 120 min at 4 °C. Tissues were then washed in PBS and incubated in solutions with progressively higher sucrose concentrations (10, 20 and 30%) over a period of 24 h. This latter procedure was to cryo-protect tissues during freezing. Whole bladders were 'snap frozen' using isopentane cooled to freezing point with liquid nitrogen and stored at −80 °C until needed. Tissue sections (7–8 μm) were cut at −18 °C and thawed onto precooled poly-lysine coated slides. Tissue morphology was examined to confirm that gross structural features were intact. Slides were air dried, wrapped in moisture resistant wrap, labeled and stored at −80 °C until used.

2.2. Tissue staining

Stored slides were removed from the −80 °C freezer, allowed to equilibrate to room temperature for 60 min, unwrapped and placed in a dry environment for a further 60 min. Slides were then washed for 3 × 5 min each in solutions of tris-buffer saline (TBS), tris-buffer saline tween (TBS-T) and TBS. Primary antibodies, diluted with 1× PBS or PBS with Triton X-100, were then put on each slide and incubated in a humidifying chamber for 18 h at 4 °C. Primary antibodies included against vimentin (vim) (1:5000; BioGenex; MU074-UC), calcitonin gene related peptide (cgrp) (1:500; Santa Cruz; sc-57053), β_1 (1:200; Santa Cruz; sc-568), vesicular monoamine transporter 2 (vmat) (1:100; Santa Cruz; sc-7721), tyrosine hydroxylase (TH) (1:2000; Abcam; ab137869). After incubation with primary antibodies, sections were washed in TBS, TBS-T, TBS wash cycles each for 20 min. Sections were subsequently incubated with the complementary secondary fluorescent antibodies: mouse, goat and rabbit primary antibodies were visualized using donkey anti-mouse/goat/rabbit IgG antibody conjugate (Molecular Probes) Alexa Fluor 488 or 594. Secondary antibodies were applied in PBS and used at 1:500 dilutions. These antibodies were applied sequentially and applied for 1 h at room temperature in a humidifying chamber in the dark. After each incubation, sections were washed 3× in TBS, TBS-T, TBS. Finally, sections were covered with Vectashield hardset mounting medium with DAPI (nucleic acid molecular probe stain) and a glass cover slip (24 × 60 mm) applied. The specificity of the vmat and β_1 antibodies were checked by pre-absorption of each antibody with its specific blocking peptide on to which it was raised: vmat blocking peptide (Santa Cruz; sc-7721P) and β_1-AR blocking peptide (Santa Cruz; sc-568P). Each antibody was incubated overnight with an excess of its specific blocking peptide (antibody:blocking peptide ratio 1:30) at 4 °C. Thereafter, the antibody solution or antibody plus peptide solution was applied to sections.

2.3. Image capture

Sections were viewed using an Olympus BX61 fluorescence microscope with ×10, ×20 and ×60 objectives. Images were captured using an Olympus XM10 monochrome camera in 16-bits digital format and examined further using Image J software (Java-based image processing program - National Institutes of Health (US)). Images taken to determine the effects of blocking peptides were taken with the same exposure as the control images in the absence of blocking peptide.

3. Results

In all bladder studies, antibodies to tyrosine hydroxylase (TH), a key element on the synthesis of noradrenaline, and to vesicular mono-amine transporter (vmat), the transporter responsible for the storage of mono-amines into synaptic vesicles, identified nerve fibers within the bladder wall. It is more than likely that these TH immunoreactive (TH-IR) and vmat immunoreactive (vmat-IR) fibers identify adrenergic nerves. However, Fig. 1 shows that the number of TH-IR fibers in the bladder wall is low and that few fibers are to be found within or between the muscle bundles of the lateral wall (Fig. 1A), or towards the base (Fig. 1B and C). Fibers, when present, tend to run singly within muscle bundles, or in small groups between the bundles (Fig. 1F and G). In contrast, TH-IR nerves were readily found associated with blood vessels, primarily in the lamina propria (Fig. 1A–E). It was only in the bladder neck, in close proximity to the urethra, that a dense adrenergic innervation was found (Fig. 1D). Thus, in the main body of the bladder there appears to be a minimal adrenergic input to the detrusor.

Towards the bladder base, muscle bundles were found occasionally with a dense innervation of TH-IR or vmat-IR fibers (Fig. 2A and B (*)). Such innervated bundles were found adjacent to bundles with no TH-IR or vmat-IR (+). The incidence of these densely innervated muscle bundles was low but this punctate innervation was seen in all bladders examined (n = 11).

Fig. 2B illustrates that there appears to be a population of cells lying between the muscle bundles that are vmat-IR (o). These cells are not nerve fibers and nuclei are readily seen in the cell bodies. Examination of the same section stained to identify TH-IR failed to show a significant or detectable staining in these cells. These vmat-IR cells were more abundant in the lateral wall near the base. Fig. 2C shows a further example of these cells (o) and a muscle bundle with TH-IR and vmat-IR nerve fibers. The cells have a stellate appearance and are typically found singly. No innervation of these vmat-IR cells by vmat or TH fibers was noted.

Further examination of these vmat-IR cells shows that they are also positive for vimentin (vim-IR) (Fig. 3A). However, within any one cell there may be a region of weaker vmat-IR, suggesting sub-cellular localization. As also shown in Fig. 3B and C not all vim-IR cells are vmat-IR suggesting that there is a sub-population of catecholamine related interstitial cells. These vim-IR and vmat-IR cells appear to form an interconnecting network of heterogeneous interstitial cells. No vmat-IR cells were seen to run within the muscle bundles. However, vim-IR cells and processes were found within muscle bundles (see also Fig. 4).

These observations suggest the possibility of the storage and release of catecholamines within the interstitial cell network. The question then arises: 'What is the target for this putative signaling system?' Since functional studies have led to the suggestion that β_1 dependent mechanisms (Gillespie et al., 2015b), rather than β_2 or β_3, are operating in the rat bladder wall, experiments were done to determine the presence and