Vascular Pharmacology 60 (2014) 84–94

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

Neurohumoral regulation of spontaneous constrictions in suburothelial venules of the rat urinary bladder

Yuki Shimizu, Satoshi Mochizuki, Retsu Mitsui, Hikaru Hashitani *

Department of Cell Physiology, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan

ARTICLE INFO

Article history:
Received 18 October 2013
Received in revised form 30 December 2013
Accepted 3 January 2014

Keywords:
Microcirculation
Bladder
Suburothelium
Vasomotion
Adrenergic nerve

ABSTRACT

Venules of the bladder suburothelium develop spontaneous phasic constrictions that may play a critical role in maintaining venular drainage of tissue metabolites. We aimed to investigate neurohumoral regulation of the spontaneous venular constrictions (SVCs). Changes in venular diameter of the rat bladder suburothelium were monitored using a video tracking system, whilst the effects of electrical field stimulation (EFS) and bath-applied bioactive substances were investigated. The innervation of the suburothelial microvasculature was examined by immunohistochemistry. EFS (10 Hz for 30 s) induced an increase in the frequency of SVCs that was prevented by phentolamine (1 μM). In phentolamine-pretreated venules, EFS suppressed SVCs with a venular dilatation in a manner attenuated by propranolol (1 μM) or L-nitro arginine (LNA, 10 μM). BRL37344 (1 μM), a β_3 adrenoceptor agonist, dilated venules and reduced the frequency of SVCs in an LNA-sensitive manner. ACh (1–10 μM) increased the frequency of SVCs. ATP (1 μM) transiently constricted venules and then caused LNA-sensitive cessation of SVCs associated with a dilatation. Substance P (100 nM) caused a venular constriction, whilst calcitonin gene related peptide (CGRP, 100 nM) caused a dilatation of venules and suppression of SVCs that were not inhibited by LNA. Immunohistochemical staining demonstrated sympathetic as well as substance P- and CGRP-containing nerves running along the venules. Spontaneous constrictions of suburothelial venules are accelerated by sympathetic α-adrenergic stimulation, but suppressed upon β-adrenergic stimulation. In addition, suburothelial venular constrictions appear to be modulated by several bioactive substances that could be released from urothelium or suburothelial sensory nerves.

1. Introduction

Overactive bladder (OAB) is a highly prevalent disorder that increases with age in both sexes. Its cause or etiology appears to be complex and multifactorial [27]. Considering the current diagnostic criteria of over active bladder, i.e., urinary urgency, enhanced signal transmission from urothelium to afferent nerves may be a primary cause of overactive bladder symptoms [35]. Normal bladder filling sensation is mediated by stretch-dependent release of ATP from urothelium that acts upon P2X3 purinoceptors located on sensory nerve endings [10,30]. ATP also acts on suburothelial interstitial cells [32] or detrusor smooth muscle cells that may modulate urothelium-afferent nerve signal transmission. Thus excessive releases of ATP could result in urinary urgency. Additionally, other bioactive substances released from the urothelium, e.g., acetylcholine [33] and nitric oxide [5], or from sensory nerves [21], e.g., substance P and CGRP, may also modulate afferent nerve activity.

The suburothelial microcirculation is critical in maintaining bladder cell function. In the normal bladder, blood flow is well maintained during the filling phase, and only transiently decreases during voiding [14]. This maintenance primarily relies on the high compliance properties of the bladder wall that allow low pressure storage of urine. However the intrinsic properties of the microvasculature including their winding arrangement and spontaneous venular constrictions (SVCs) [15,16] may also be beneficial in maintaining bladder microcirculation during storage phase.

It has been proposed that overactive bladder results from ischemia and/or reperfusion [9] associated with bladder outlet obstruction, aging or atherosclerosis [2,3,34]. Indeed, alterations in urothelial mediated regulation in ischemic bladders result in detrusor instability [2–4]. Previous studies focused on the arteries/arterioles in terms of their function in supplying oxygen and nutrients to the bladder, whilst the role of venules in regulating the bladder microcirculation has garnered less attention. Of studies that examine bladder venules, it is suggested that diminished venular drainage can result in tissue metabolite accumulation and contribute to 'urinary urgency' upon acidification-induced stimulation of TRP channels at sensory nerve endings [8,20]. Thus it is reasonable to assume that improvement of bladder storage symptoms with α-adrenoceptor antagonists [22] or PDE5 inhibitors [25] may be attributed to their vasodilator action on the suburothelial microvasculature.

Abbreviations: LNA, N-nitro-L-arginine; SIN-1, 3-(4-Morpholinyl)sydnonimine; CGRP, calcitonin gene-related peptide; EFS, electrical filed stimulation; SVC, spontaneous venular constriction; OAB, overactive bladder.

* Corresponding author. Tel.: +81 52 8538131; fax: +81 52 8421538.
E-mail address: hasitani@med.nagoya-cu.ac.jp (H. Hashitani).

1537-1891/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.vph.2014.01.002

β_3-Adrenoceptor agonists may also exert an influence by improving the microcirculation within the bladder wall [1]. For example, mirabegron, a β_3-adrenoceptor agonist, reduces bladder hyperactivity in a rat model of chronic bladder ischemia [28].

The suburothelial microvasculature runs within the connective tissue layer just beneath the urothelium, where both efferent and afferent nerves are abundant, suggesting their function could readily be affected by neurohumoral substances released from nerves as well as the urothelium. We aimed to investigate the neurohumoral regulation of SVCs in the rat bladder since any imbalance between excitatory and inhibitory vasoactive substances may result in suburothelial microcirculation dysfunction. Agents that improve the microcirculation are potential tools for the treatment or even prophylactic treatment of overactive bladder.

2. Materials and methods

2.1. Animal and ethical approval

Male Wistar rats 6 to 8 weeks old were used throughout this study. They were housed, cared for and acclimatized (before the experiments). For experiments, rats were anesthetized and exsanguinated by decapitation according to procedures approved by the Nagoya City University Medical School Experimental Animal Committee.

2.2. Tissue preparation

The bladder was removed, cut open and pinned in a dissecting dish with the urothelial side uppermost. The mucosal layer was dissected away from the detrusor smooth muscle layer, and the urothelium was carefully removed using ophthalmology scissors leaving the suburothelial layer.

2.3. Video imaging

Suburothelial preparations were superfused with warmed (36 °C) physiological salt solution (PSS) at a constant flow rate (about 2 ml/min). Venules were readily distinguished from arterioles by their larger diameters and thinner walls as well as their regularly occurring SVCs. Changes in the diameter of venules were recorded with a video camera, and analyzed using Diamtrak, video edge detection software. Electrical field stimulation (EFS) was applied to preparations by passing brief currents (10 Hz, pulse width 50 μs) between a pair of platinum electrodes in the recording chamber. The neural selectivity of evoked responses was confirmed by their sensitivity to tetrodotoxin (1 μM).

2.4. Intracellular calcium imaging

Suburothelial preparations were incubated in low Ca^{2+} PSS ($[Ca^{2+}]_o = 0.1$ mM) containing 1 μM fluo-8 AM (AAT Bioquest, Sunnyvale, CA, USA) and cremphor EL (0.01%, Sigma) for 30 min at 35 °C. Following incubation, preparations were superfused with dye-free PSS and illuminated at 495 nm with fluorescence emissions (above 515 nm) captured as described previously [10,11]. Relative amplitudes of Ca^{2+} transients were expressed as $\Delta F_t/F_0 = (F_t - F_0) / F_0$, where F_t is the fluorescence generated by an event, and baseline F_0 is the basal fluorescence.

2.5. Immunohistochemistry

Suburothelial whole mount preparations were immersed in fixative containing 2% formaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer (pH 7.4) for 10 min, and then further immersed in the same fixative overnight at 4 °C. The fixed specimens were washed in dimethyl sulfoxide (DMSO) and then in PBS.

Specimens were incubated with PBS containing 0.3% Triton X-100 for 10 min, incubated with Block Ace (Dainippon Seiyaku) for 20 min and incubated with primary antibodies diluted in PBS containing 2% bovine serum albumin and 0.3% Triton X-100 for 4 days at 4 °C. Primary antibodies used in the present study were as follows: mouse monoclonal antibody for α smooth muscle actin (αSMA; 1:500, clone 1A4, Sigma), rabbit anti tyrosine hydroxylase (TH) antibody (1:1000, Millipore), rabbit anti-SP antibody (1:1000, Immunostar) and rabbit anti-CGRP antibody (1:50, Progen Biotechnik). Specimens were then incubated with biotinylated swine anti-rabbit IgG antibody (1:300, Dako) for 30 min at room temperature. Specimens were incubated with Alexa 488-conjugated streptavidin (10 μg/ml, Jackson ImmunoResearch) and Cy-3-conjugated goat anti-mouse IgG antibody (2.5 μg/ml, Millipore) for 2 h at room temperature and coverslipped with a fluorescence mounting medium (Dako). Whole mounts were examined using a confocal laser scanning microscope (LSM 5 PASCAL, Zeiss).

2.6. Solutions and drugs

The composition of PSS was (in mM): NaCl, 120; KCl, 4.7; $MgCl_2$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 15.5; KH_2PO_4, 1.2 and glucose, 11.5. The pH of PSS was 7.2 when bubbled with 95% O_2 and 5% CO_2, and the measured pH of the recording bath was approximately 7.4.

The drugs used were acetylcholine (ACh), ADP, ATP, αβ-methylene ATP (αβ-MeATP), $BaCl_2$, BRL37344, human CGRP, phentolamine, propranolol, N-ω-nitro-L-arginine (LNA), 3-(4-morpholinyl)sydnonimine (SIN-1), substance P and tetrodotoxin. All drugs were dissolved in distilled water except SIN-1 which was dissolved in ethanol. The final concentration of solvents in PSS did not exceed 1:1000.

2.7. Calculations and statistics

Measured values are shown as the mean ± SD. Statistical significance was tested using the paired *t* test with $P < 0.05$ considered significant. The number of tissues is denoted 'n', whilst the number of animals is denoted 'N'.

Peak amplitude of SVCs was measured as the value from the basal diameter to the peak of constrictions. Frequency was calculated as an average during 3 min either prior to (control) or during drug application, or was calculated during EFS.

3. Results

3.1. General observations

Consistent with our previous report [15], suburothelial venules developed spontaneous phasic constrictions. In 54 suburothelial venules (n = 54, N = 48), SVCs were associated with a transient reduction in their diameter to 78.5 ± 13.2% of their resting diameter (70.9 ± 24.9 μm) and occurred at a frequency of 4.7 ± 1.7 min^{-1}.

During preliminary experiments, a substantial delay between EFS and the onset of venular constrictions were evident. Therefore, the time course of nerve-evoked responses in venules and arterioles was compared by visualizing their Ca^{2+} transients. EFS (10 Hz for 1 s) triggered a prompt Ca^{2+} transient in nerve fibers that was immediately followed by Ca^{2+} transients in the arteriolar smooth muscle cells with a delay of 0.57 ± 0.04 s (n = 5, N = 4, Fig. 1A, B). Subsequently, EFS-induced Ca^{2+} transients in venular smooth muscle cells were observed with a delay of 2.1 ± 0.17 s (n = 5, N = 4, Fig. 1A, B, Supplement movie 1).

Suburothelial venules were immunoreactive for α smooth muscle actin (αSMA, Fig. 1C a, b, d), whilst arterioles showed relatively weak αSMA immunoreactivity (Fig. 1Ca, b). Both arterioles (Fig. 1Cb, c) and venules (Fig. 1Cb, c) were innervated, and varicose sympathetic nerve fibers immunopositive for tyrosine hydroxylase (TH) run along the microvasculature as well as transversely over venules (Fig. 1Cd, e).