INTRODUCTION

Urinary bladder unmyelinated afferent C-fibres are involved in micturition reflex regulation both under normal conditions and during pathophysiological processes (e.g. bladder overactivity - OAB) (1, 2). Detrusor overactivity (DO) is defined as involuntary detrusor contractions (spontaneous or provoked) during filling (3). When we use the term DO we mean involuntary bladder behavior observed in rats with bladder infused with hyperosmolar saline solution, although we recognize that this condition is quite distinct from true DO as defined by the International Continence Society and seen in the human condition. Emerging evidence implies that the transient receptor potential ion channel of the vanilloid (TRPV1) and ankyrin (TRPA1) may play a pivotal role in the development of DO (4, 5). TRPV1 receptors are co-expressed with TRPA1 receptors on sensory C-fibres within the urothelium, suburothelial space, and muscle layer as well as around blood vessels throughout the rat urinary bladder. Additionally, these receptors have also been detected in urothelial cells (6, 7). Many conditions may cause the urine hyperosmolarity, as follow: high glucose amount and/or potassium concentration in urine, etc. (8). Osmotic changes influence on TRPV receptors activity (9). Zhang et al. (10) found that TRPA1 mediates an osmotically-activated ion channel and is important in mechanosensation. Hyperosmolar urine may activates afferent C-fibres directly and/or indirectly via urothelium acting on TRPV and TRPA1 receptors leading to DO. Hyperosmolarity determines the local effector function of bladder afferent C-fibres by changes in vanilloid receptors activity. Hypertonic urine may penetrate submucosal layers of urinary bladder and activates capsaicin-sensitive C neurons and consequently, induce neurogenic inflammation leading to OAB (9, 11). Kulick et al. concluded that the 16 hours of water deprivation proved sufficient to determine urine concentrating ability of kidneys. The urine concentration tests in female rats revealed that mean urine osmolarity was 2080 mOsm/l (12). Previous study revealed that hypertonic saline within physiological osmolarity range induce concentrated-dependent OAB. The urinary bladder overactivity can be induced by continuously intravesically infusion of hypertonic saline solution (2080 mOsm/l) at a rate of 0.046 ml/min. (13). The study aims to investigate some of the mechanisms through which the hyperosmolarity induced urinary bladder overactivity. We compared the bladder motor and sensitive activity in response to partial and complete blockade of TRPV1-6 and TRPA1 receptors by using the non-selective TRP inhibitor ruthenium red and the selective TRPV1 agonist (capsaicin).

EFFECT OF PARTIAL AND COMPLETE BLOCKADE OF VANILLOID (TRPV1-6) AND ANKYRIN (TRPA1) TRANSIENT RECEPTOR POTENTIAL ION CHANNELS ON URINARY BLADDER MOTOR ACTIVITY IN AN EXPERIMENTAL HYPEROSMOLAR OVERACTIVE BLADDER RAT MODEL

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The study investigated the mechanisms through which the hyperosmolarity might induce detrusor overactivity (DO). We compared the bladder activity in response to partial and complete blockade of TRPV1-6 and TRPA1 receptors. Experiments were performed on 42 rats. DO was induced by using hyperosmolar saline. All animals were randomly divided into six groups. The measurements represent the average of five bladder micturition cycles. Hyperosmolar saline induced DO. The complete blockade of TRPV1-6 and TRPA1 prevented DO. The partial blockade of TRPV1 didn't prevented DO. In the voiding phase periodical bladder contractions complexes occurred leading to slow urine flow due to bladder distension. Ruthenium red and capsaicin resulted in complete disorganisation of detrusor muscle contractility impairing urine voiding and leading to constantly lasting urine retention in healthy rats. Conclusions: hyperosmolar-induced DO is mediated by TRPV and TRPA1 channels; the hyperosmolar stimuli of urinary bladder might be transmitted mostly via ruthenium red sensitivity pathway.

Key words: capsaicin, detrusor overactivity, hyperosmolarity, ruthenium red, transient receptor potential ankyrin, transient receptor potential vanilloid type 1
MATERIAL AND METHODS

Animals

Experiments were performed on 42 adult female Wistar rats (weight: 200-275 g). Rats were housed individually in cages. The animal room was maintained at a constant temperature of 23°C, humidity and a 12:12 hrs alternating light-dark cycle. They were fed with animal food (Labofeed; Keynia, Poland) without any restriction to water. The study has been approved by the Local Animals Ethical Committee.

Experimental groups

Forty two animals were divided randomly into six groups: I (control) - healthy rats (n=12), II - rats with hyperosmolar induced DO (n=6), III - rats with hyperosmolar induced DO and intravesical instillation of 100 µM ruthenium red solution (n=6), IV - rats with hyperosmolar induced DO after intravesical instillation of 1 mM capsaicin solution (n=6), V - healthy rats with intravesical instillation of 100 µM ruthenium red solution (n=6), VI - healthy rats after intravesical instillation of 1 mM capsaicin solution (n=6).

Anaesthesia

All the surgical procedures and urodynamic studies were performed under anaesthesia using intraperitoneal injection of 1.2 g/kg urethane (Sigma-Aldrich, St. Louis, USA) (11) and in the case of capsaicin the intravesical instillation was at a dose of 0.4 g/kg.

Hyperosmolar model of overactive bladder (OAB)

The urinary bladder overactivity was induced by continuously intravesically infusion of hypertonic saline solution (2080 mOsm/l) at a rate of 0.046 ml/min., as previously described (13).

Administration of drugs

The following drugs were used: ruthenium red (Adeviq-SPIN, Poland) and capsaicin (Sigma-Aldrich, Germany). Ruthenium red - non-selective TRP inhibitor: TRPV1-6 and TRPA1 (final concentration 100 µM) was dissolved in 0.9% saline. Capsaicin - selective TRPV1 agonist leading to complete TRPV1 desensitisation (final concentration 1 mM) was dissolved in special solution composed of 0.9% saline (80%), absolute ethanol (10%) and Tween 80 (Sigma-Aldrich, Germany) (10%) (the volume participation of individual components in solvent expressed in the per cent, is provided in square brackets). Under urethane anaesthesia, the bladder was catheterized through the urethra and emptied. A volume of 0.3 ml of 1 mM capsaicin was injected through the catheter (group IV and VI) at a rate of 0.15 ml/min. Capsaicin was left in contact with the mucosa for 15 minutes. The bladder was emptied again and flushed out using 0.5 ml 0.9% saline at a rate of 0.15 ml/min. (14, 15).

Surgical procedure

Bladder catheter implantation: under urethane anaesthesia, the abdomen was opened through a midline incision and the bladder end of the polyethylene catheter (o.d. 0.97 mm/ i.d. 0.58 mm; BALT, Poland) was passed through a 1 mm incision at the apex of the bladder dome and secured in place by a silk ligature 4-0, as previously described (15).

Urodynamic studies

Cystometry was performed under urethane anaesthesia after a 1 h recovery period from the surgical procedure. A room temperature solution was infused at a rate of 0.046 ml/min. continuously into the bladder. The free end of the implanted catheter was connected via a T-stopeck to a pressure transducer (UIF, MorroBay, CA, USA) and injection pump (Unipan340A, Poland). Cystometry was recorded using ML110-BridgeAmp (ADInstruments, Australia) hardware and PowerLab®SSP (ADInstruments, Castle Hill, Australia) software, as previously described (15, 16).

Study protocol

All animals underwent cystometry using isoosmolar (308 mOsm/l) saline solution (group I), hyperosmolar (2080 mOsm/l) saline solution (group II), hyperosmolar (2080 mOsm/l) saline and 100 µM ruthenium red solution (group III), hyperosmolar (2080 mOsm/l) saline solution in 24 hours after intravesical 1 mM capsaicin instillation (group IV), isoosmolar (308 mOsm/l) saline and 100 µM ruthenium red solution (group V) or isoosmolar (308 mOsm/l) saline solution in 24 hours after intravesical 1 mM capsaicin instillation (group V). Additionally, the influence of capsaicin's vehicle on bladder function was urodynamically tested, however there was no significant influence on bladder function as compared to control group the further analysis and results were omitted.

The measurements repeated in each animal represents the average of five bladder micturition cycles, after obtaining repetitive voiding. The following cystometrogram parameters were recorded: BP - basal pressure (cmH2O), PT - threshold pressure (cmH2O), MVC - micturition voiding pressure (cmH2O), ICI - intercontraction interval (min.), compliance (ml/cmH2O), MI - motility index (cmH2O x s/ml), MI - detrusor overactivity index (cmH2O/ml) in group I and DOI - detrusor overactivity index (cmH2O/ml) in group II - IV, depicted as quotient of the sum of amplitudes of all detrusor contractions during filling phase and functional bladder capacity (16). MI is defined as the enclosed area between the sampled data and their minimum on the selected interval. MI which is dependent on basal pressure, contractions profile and also compliance of detrusor could be useful for bladder motor activity estimation. Motility index is generally used for contractile activity evaluation of smooth muscle (16).

Statistical analysis

The results are expressed as mean and standard deviation (±S.D.). The Kruskal-Wallis test was used to compare between groups and "post hoc" multiple comparison tests for statistically significant results. Statistical significance was set at p<0.05 for all tests. Additionally, the changes of CMG parameters (mean values) were shown in percentage as compared to referral group. The values of group II was referred to group I, as well as the group III and IV to group II.

RESULTS

Effect of intravesical instillation of hyperosmolar saline solution on urinary bladder motor activity in normal rats (group I and II)

Intravesical infusion of hyperosmolar (2080 mOsm/l) saline solution induced detrusor overactivity. All hyperosmolar DO rats did not exhibit macroscopical signs of bladder inflammation, i.e.
redness, oedema as well as wall thickening, mucosal erosions, ulcerations, petechial haemorrhages on the serosal surface. Cystometric evaluations revealed a significant decrease of intercontraction intervals (51%) and functional bladder capacity (50%). Additionally, an increase of basal pressure (118%), detrusor overactivity index (413%) and motility index (33%) were observed (Table 1, Fig. 1A,B, Fig. 2). No statistical differences of threshold, micturition voiding pressure and compliance were obtained.

Effect of intravesical instillation of ruthenium red and capsaicin on urinary bladder motor activity in rats with hyperosmolar detrusor overactivity (group III and IV)

Simultaneously intravesical administration of 100 µM ruthenium red with hyperosmolar (2080 mOsm/l) saline solution prevented from hyperosmolar detrusor overactivity development (Table 1, Fig. 1C, Fig. 2). In comparison with hyperosmolar DO rats we observed, statistical significant increase of intercontraction interval (181%), functional bladder capacity (178%), and compliance (235%). Also statistical significant decrease of basal pressure (36%), micturition voiding pressure (25%), detrusor overactivity index (86%) and motility index (44%), were observed. The difference of threshold pressure was not statistically significant.

In contrast, intravesical infusion of hyperosmolar (2080 mOsm/l) saline solution 24 hours after 1 mM of capsaicin treatment revealed polymorphic micturition cycles with improper storage and voiding phase leading to bladder distension (Table 1, Fig. 1D, Fig. 2). The short storage phase was characterised by an increased intravesical pressure (higher basal and threshold pressure). During voiding phase periodical bladder contractions complexes occurred leading to slow flow of urine (low volume) causing incremental bladder distension. In comparison with ruthenium red instillation, the amplitude of NVCs and intravesical pressure were almost doubled after capsaicin treatment. In both groups (V and VI), the voiding phase lacked proper detrusor contractility (generation of micturition voiding pressure - MVP). Nevertheless, as a consequence of the lack of periodically generated MVP and incomplete bladder emptying, persistent urine retention occurred. In cases where maximal cystometric capacity was achieved associated with bladder distension we recorded a constant, dripping flow of urine through the urethra. Because there were no proper micturition cycles, bladder activity analysis was based on a motility index (defined as the enclosed area between the sampled data and their minimum on the selected interval), which revealed no statistical differences between all groups (I, V and VI).

**DISCUSSION**

The types and some pathomechanisms of lower urinary tract symptoms (LUTS) are well described. Such symptoms are prevalent and burdensome, therefore the patients change their fluid intake behavior to avoid the LUTS occurrence. However, there is a lack of data about how individuals with LUTS manipulate their fluid intake. Elstad et al. described the fluid manipulation in patients with LUTS based on a population based,
random sample epidemiologic survey of urologic symptoms of 5503 patients from Boston Area Community Health Survey (17). The observations revealed that some patients restricted fluid intake while others increased it, in both cases with the expectation of improved LUTS. Fluid intake was greater in men and women with frequency. While women with frequency drank significantly more water, as compared to women with urgency. These results indicate the divergent expectations of the role of fluids in alleviating LUTS, lead some individuals to restrict and others to increase fluid intake. Additionally, Hashim et al. (18) in their study concluded that there is increasing evidence suggested that the fluid intake reduction can improve OAB symptoms, with a general consensus that caffeine reduction may be beneficial. However, further clinical trials are needed on fluid manipulation in terms of the types of fluids drank, the timing of fluid intake, and the effects of exercise and outside ambient temperature on OAB symptoms. In clinical practice, many patients complain that fluid intake restriction increase the severity of LUTS. The

Fig. 1. Cystometrogram's traces in healthy rats (A), rats with hyperosmolar DO (B), rats with hyperosmolar DO with 100 µM of ruthenium red instillation (C), rats with hyperosmolar DO after 1 mM of capsaicin treatment (D), healthy rats with 100 µM ruthenium red instillation (E) and healthy rats after 1 mM capsaicin treatment (F). Bladder distension means incomplete bladder emptying with persistent urine retention. The figure shows 20-minutes (A-C) or 42-minutes interval (D-F) - horizontal axis. Vertical axis estimates intravesical pressure of (-5) - (25-40) cmH₂O range.
we explored the effect of partial and complete blockade of these receptors by ruthenium red and capsaicin in hyperosmolar stimuli (2080 mOsm/l) we observed, a statistically significant increase of ICI, fBC, and compliance, as well as decrease of BP, MVP, DOI, and MI. Such changes may result from the complete suppression of C-fibres and secondarily an impaired inflammatory neurogenic response to hyperosmolar stimuli. Surprisingly, the values of ICI, fBC were greater and DOI, MI were smaller when compared to healthy rats. Complete blockade of TRPV and TRPA1 receptors also decreased bladder motor activity in comparison with naive rats. This suggested that these receptors are important during mechanical transduction in the micturition process.

In contrast, the partial blockade of TRPV1 by 1 mM capsaicin did not prevent DO development in hyperosmolar DO rats. Polymorphic micturition cycles with a statistically significant increase of BP, PT, MI, and decrease of ICI, fBC, compliance, and DOI were obtained. The short storage phase was characterised by increased intravesical pressure (higher BP and PT). During the voiding phase periodical bladder contractions complexes occurred leading to impaired urine flow due to the bladder distension increment. We postulate that this might be the result of overstimulation of TRPA1 and TRPV (except TRPV1) receptors. Tramontana et al. revealed that superfusion of slices of the rat urinary bladder with hypertonic NaCl produced a remarkable and concentration-dependent increase in the outflow of CGRP. This effect was completely abolished by pre-exposure of the tissue to capsaicin (19). In contrast Del Bianco et al. (27) observed that hypertonic NaCl-evoked CGRP release from capsaicin-sensitive sensory nerves was not affected by ruthenium red. Also, activation of TRPA1 receptors on capsaicin-sensitive afferents (containing SP and CGRP) and urothelial cells induces DO (6).

Ruthenium red and capsaicin caused complete disorganisation of detrusor muscle contractility hindering proper urine voiding and leading to persistent urine retention in healthy rats. Increased spontaneous detrusor overactivity occurred during the storage phase of the micturition cycle. Phasic detrusor contractions of low amplitude with accompanying increased intravesical pressure occurred. In comparison with ruthenium red instillation, the changes were almost doubled after capsaicin treatment. Also, Komiyama et al. reported the inhibition of rhythmic bladder contractions after capsaicin and resiniferatoxin instillation in normal and chronic spinal cord-injured rats (28).

The complete blockade of TRPV1-6 and TRPA1 receptors by 100 µM ruthenium red prevented hyperosmolar-induced development of DO. In comparison with 'hyperosmolar DO' rats we observed, a statistically significant increase of ICI, fBC, and compliance, as well as decrease of BP, MVP, DOI, and MI. Such increment of urine concentration due to water restriction seems to play a potential role in the development of LUTS. As we mentioned above, the patients with urgency restrict the fluid intake to avoid the symptom. It is known that urgency appears when detrusor contractions occur. Therefore, the fluid restriction in out of sense leading to pathological "vicious cycle", because of its induce urine hyperosmolarity and secondarily detrusor overactivity, as well as LUTS symptoms alteration. The observations described in our animal model of OAB show that it seems to be highly recommended to provide the clinical evidence of urine concentration and LUTS symptoms.

Transient receptor potential ion channels family may be subdivided into six main subfamilies: TRPV (vanilloid), TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPP (polycystin), and TRPML (mucolipin) (20). TRPV1, TRPV4, and TRPA1 receptors present on urothelium and bladder primary sensory neurons, and are involved in somatosensory processes, including the transduction of chemical, thermal, and mechanical stimuli (6, 20, 21). The detrusor overactivity is strictly correlated with urothelial activity and the local effector function of afferent C-fibres causing neurogenic inflammatory response, as a result of substance P (SP) and calcitonin gene-related peptide (CGRP) release (22, 23). Neurogenic inflammation can be induced by cyclophosphamide treatment leading to DO development (24). The results of Bossowska et al. (25) study revealed that urinary bladder sensory nerves contained mostly SP and CGRP in porcine. It seems likely that hyperosmolar urine may lead to greater excitability of bladder afferent nerves and muscle cells, as a result of bladder urothelium activity changes inducing DO. Moreover, the spontaneous calcium oscillations in smooth muscle cells seem to underlie the motor urinary bladder activity (26).

In response to hyperosmolar stimuli we observed bladder motor overactivity development, characterized by a significant decrease of ICI, fBC with an increase of BP, DOI, MI, and non-voiding contractions frequency. Although, experimental evidence proved that concentrate-dependent hyperosmolar stimuli induce detrusor overactivity in rats, the pathophysiological mechanism has not yet been established (13). Furthermore, to investigate the importance of vanilloid (TRPV) and ankyrin (TRPA1) receptors for hyperosmolar-induced OAB, we explored the effect of partial and complete blockade of these receptors by ruthenium red and capsaicin in hyperosmolar-induced DO, as well as in healthy rats.
Our observations suggest a role for TRPV and TRPA1 mediated pathways in motor functions of the urinary bladder under naive conditions, and also in response to hyperosmolar stimuli.

CONCLUSION

Hyperosmolar-induced detrusor overactivity is mediated by vanilloid (TRPV) and ankyrin (TRPA1) transient receptor potential ion channels. The hyperosmolar stimuli of urinary bladder might be transmitted mostly via a ruthenium red sensitivity pathway. Additionally, the TRPV and TRPA1 receptors seem to be involved in the urinary bladder afferent mechanosensory and hyperosmotic transduction.

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