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Piezo1-dependent inhibition of background potassium current in the rat bladder smooth muscle cells

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The smooth muscle layer of the urinal bladder (detrusor), along with the urothelium, has autonomous mechanosensitivity and serves as a main sensitive receptor in the organ. Piezo1, together with TREK-1 channel, could play a role of local mechanoreceptors in bladder detrusor smooth muscle (DSM) cells. Piezo1 is a so-called "true" mechanosensitive calcium-permeable ion channel, sensitive to pressure, shear stress, and is activated by pharmacological agonist Yoda1. Using patch-clamp and microfluorescence calcimetry, an unexpected effect from Yoda1 was shown, that is inhibition of rest K^+ currents at depolarizing command voltage up to +80 mV. The functional presence of Piezo1 is confirmed by Yoda1-induced rise of intracellular Ca^{2+} concentration in DSM cells, this was visualized using a Ca^{2+} sensitive dye Fluo-4 AM, and polymerase cyclic reaction with reverse transcription. In conclusion, Piezo1 channels present in DSM cells and are selectively activated with Yoda1, that causes the inhibition of resting potassium currents.
Key words: Piezo1; Yoda1; smooth muscle cells; bladder; potassium currents.

INTRODUCTION

In the urinary bladder, shear stress and stretching are the main stimulation factors due to its functional role in collecting and holding urine. Although the mechanism of mechanosensation in the smooth muscle layers of the bladder is still not fully explored, it is already known that TREK-1 mechanosensitive two-pore domain K^+ ion channel is functionally present in DSM cells that cause depolarization in case of stretching [1]. Further piece of evidence suggests an expression of other mechanosensitive ion channels from Piezo- and TRP- ion channel families [2, 3]. Piezo1 in contrast to Piezo2 and TRP channels is spreading in different types of "non-sensitive" tissues such as endothelium, cartilages, alveoli, red blood cells, smooth muscle cells from vessels, interstitial cells of Cajal (ICC) and other origins [4–7], as well as in bladder detrusor [2]. But at the same time, the functional presence of Piezo1 in DSM cells is still questionable.

Piezo1 channel is one of the so-called "true" mechanosensitive ion channels, which has mechanosensitivity as a major function and which was explored in the last decade [8]. It has one monomer – PIEZO1, three of them together compose a nonselective mechanosensitive channel that conducts mostly cations with a preference for Ca^{2+} and Na^+ with a single-channel conductance of 29 pS [6]. The mechanism of mechanosensation of the Piezo1 is still not fully understood. Yet, it is not dependent upon the cytoskeleton and the extracellular matrix as was shown in the work with a Piezo1 and bilayer lipid drops [9]. Several chemical agonists of Piezo1 are known: Yoda1 and Jeday1, activation of which can be blocked with antagonist – Dooku1 through direct competition to the linking site [10]. Yoda1(2-(5-{[(2,6-Dichlorophenyl)methyl]sulfanyl}-1,3,4-thiadiazol-2-yl)pyrazine) as a small synthetic chemical compound has been shown as a highly selective agonist, with activation specifically only Piezo1 and not Piezo2 despite their similarity [11]. Piezo1 channel can be sensitized through the bradykinin and cAMP receptors – Epac1 by the activation of PKA and PKC [6].

Piezo1 is the one of the most promising future therapeutic target for mechanosensitive-related disorders of the urinary bladder and other related diseases [12].

The goal of this research is to elucidate the functional presence of Piezo1, confirm the connection of inhibition of K^+ currents with the increase in intracellular calcium concentration $[Ca^{2+}]_{in}$ because of Piezo1 activation, and identify the signaling pathways that are involved in this process in isolated DSM cells of Wistar rats.

METHODS

DSM cells were isolated from the bladders of 10 three-month-old male Wistar rats. Decapitation of rats were performed under CO_2 anesthesia and in accordance with relevant guidelines of bioethics of Ukraine for laboratory animals and with the standards outlined in the International principles of the European Convention for the Protection of animals, used for experimental purposes (EU Directive 2010/63/EU). The experimental protocol received approval from the Bioethics Committee of the Bogomoletz Institute of Physiology (BIPh) under (Permission No. 2/23 from 12.07.2023). Animal husbandry and care was conducted by the vivarium department of the Bogomoletz Institute of Physiology. Isolated bladders were placed in ice-cold nominally Ca^{2+}/Mg^{2+}-free Sodium glutamate solution containing (in mmol/l): 102 - NaCl, 40 - sodium glutamate, 5 - KCl, 10 - HEPES, 5 - D-Glucose (pH adjusted to 7.35 with NaOH). The urothelial layer and connective tissue were removed surgically. Tissue pieces (1×1 mm) were processed with 2 step isolation protocol. The first step of preparation was performed in Ca^{2+}/Mg^{2+}-free Sodium glutamate solution with 1 mg/ml bovine serum albumin (BSA), 1 mg/ml dithiothreitol (DTT), and 1 mg/ml papain (all reagents above from "Sigma", USA) at 37°C for 25 min. Then the tissue was pipetted, centrifuged (1000 rpm, 4 min), and washed with Ca^{2+}/Mg^{2+}-free Sodium glutamate solution with an additional 100 μmol/l $CaCl_2$. The second step contained 1 mg/ml BSA, 1 mg/ml Trypsin inhibitor, and 1 mg/ml Collagenase from *Clostridium histoliticum* Type II ("Sigma", USA), and incubated at 37°C for 20 min. After that, the cell suspension was pipetted, centrifuged (1000 rpm, 4 min), and washed with fresh 100 μmol/l $CaCl_2$ Sodium Glutamate solution.

ICC-like cells from detrusor have been selected from cell suspension according to their typical morphology in the muscle layer as described: elongated cells with several branches or stellate cells [13].

Whole-cell patch-clamp was performed with freshly isolated cells, which were transferred into 1 ml of Tyrode's solution containing (in mmol/l): 142 - NaCl, 5 - KCl, 2 - $CaCl_2$, 1 - $MgCl_2$, 10 - HEPES, 5 - D-Glucose (pH adjusted to 7.35 with NaOH). For adhesion to the plastic Petri dish cells were left for at least 30 min at 20-25°C before the experiment started. Patch pipettes (WPI, pulled on P-1000 puller ("Sutter Instrument", USA) and fire-polished) had resistance of 3-5 MΩ when filled with an internal solution containing (in mmol/l): 81 - Na-Gluconate, 40 - KCl, 8 - EGTA, 10 - HEPES, 5 - $CaCl_2$, 1 - $MgCl_2$, 2 - Mg-ATP (pH adjusted to 7,35 with NaOH). Patch-clamp was performed using a PC-ONE amplifier ("Molecular Devices LLC", USA), and data was recorded and stored with pClamp 8.0 software ("Axon Instruments", USA), pipette capacitance and series resistance were compensated. The electrophysiological protocol was designed to measure the $I\sum$ at stationary command potentials (V_{comm}) of –100 mV and +80 mV and construct the I-V curve between these values. Stimulation was repeated every 3 seconds, resting potential of – 40 mV. For every recording 5 control traces were made for null subtraction. The 20 μmol/l Yoda1 solution was applicated directly onto the cells by using the flow application system (stock solution 10 mmol/l in DMSO ("Sigma", USA). The final DMSO concentration at work is less than 0.02%). In control experiments from 0.02 to 0.2% DMSO did not affect cell currents.

For registration of Yoda1 derived Ca^{2+} influx in DSM cells the dynamic calcimetry

with highly Ca^{2+} sensitive fluorescent dye Fluo-4 AM (494/506 nm, "Invitrogen", USA) in DMSO (final DMSO concentration less 0.002%) was used. The cell suspension was loaded in the bath with covering glass (0.1 mm thick) and left for at least 40 min for good adhesion. For the dye loading, bath with isolated cells was incubated for 30 min in the dark at room temperature (2 μl of 50 nmol/l dye stock in 1 ml Tyrode's solution). After loading cells were washed for 10 min in the dark with fresh Tyrode's solution. Dynamic calcimetry visualization was performed with Zeiss LSM 5 PASCAL VarioTwo laser scanning confocal system with Axiovert 200 microscope and 40X objective ("Zeiss", Germany). Cell stimulation was made by a 200 mW Ar-ion laser ("Zeiss", Germany). For cells stimulation with 20 μmol/l Yoda1 and buffer exchange, a flow stimulation system was used. Data was collected and primary analyzed by the standard software PASCAL.

Analysis of patch-clamp recordings was performed in ClamFit 10.7 software ("Axon instruments", USA). For time sweep presentation 10 ms signals were averaged at V_{comm} = –100 mV and V_{comm} = +50 mV. The diversity of cell size was normalized to their capacity. The amplitude of $[Ca^{2+}]_{in}$ signals was quantified as a ratio of the mean of peak fluorescence (F) in the origin of interest (ROI) to the background fluorescence level (baseline, F_0). Final data analysis was performed with Welch Two Sample T-test and R programming language (version 2023.03).

To validate the expression of the PIEZO1 in the urinary detrusor in Wistar rats, PCR with reverse transcription was performed. To detect the PIEZO1 whole sample mRNA was extracted as described elsewhere [14]. Generation of cDNA and further PCR were performed using a one-step RT-PCR kit ("Ltd BioCorp", Ukraine). Following primers (synthesized by "Metabione", Germany) were used: Piezo1 (F - 5'-CGCAACCTCACGGGCTTC-3', R - 5'-GGGCCTCGCTCACTGTATCC-3'), TREK-1 (F - 5'-GTGGAGGAGACATTTATTAAGT-3', R - 5'-GAAGAGGAGACAGCCAAACA-3'), TRPV4 (F - 5'-TTTGCTCTTATTTCTACTCC-3', R - 5'-GCTGGCTTAGGTGACTCC-3'), Piezo2 (F - 5'-ACCGACCCCAATAGTTGCTT-3', R - 5'-GCATCCGTGAGAGGAAAAGA-3') and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control (F - 5'-CAA CTCCCTCAAGATTGTCAGCAA-3', R - 5'-GGCATGGACTGTGGTCATGA-3'). Gel electrophoresis was performed with a Powerpac kit ("BioRad", USA) on 1.5% agarose ("Sigma", USA) gel with Ethidium bromide as DNA marker ("Sigma", USA) in TBE buffer made from 5X mix ("Sigma", USA).

RESULTS

It was previously shown that DSM cells functionally present Potassium mechanosensitive ion channel TREK-1 [1]. RT-PCR results show that in detrusor there are also several Ca^{2+}-conductive mechanosensitive ion channels expressed (Fig. 1). Piezo1 expression in detrusor is lower because its band is less bright than in the urothelium, although TREK-1 band is less bright in the urothelium, which is normal as urothelium contains *Lamina propria* and muscularis mucosae with smooth muscle cells in it [15].

Piezo1 is known to be the only genuine mechano-gated ion channel for whom a

reliable chemical activator, Yoda1, was discovered. Unexpectedly, application of 20 μmol/l Yoda1 under the whole-cell patch-clamp didn't activate inward cation currents, typical for Piezo1 activation, but caused inhibition (at 60 ±30 pA with rest currents 210 ± 45 pA) of typical for DSM cells [16] K^+ outward currents at V_{comm} +80mV (n = 11) (Fig. 2).

In contrast, a similar experiment in the whole-cell patch-clamped isolated ICC-like cells has shown that the same concentration of Yoda1 is capable of producing a classical Piezo1-mediated response described in the literature [7]. Thus, Piezo1 activation of ICC-like cells causes a drastic increase of currents at positive and negative V_{comm} and close to zero millivolt reversal potential, which is similar to typical Piezo1 currents (Fig. 3).

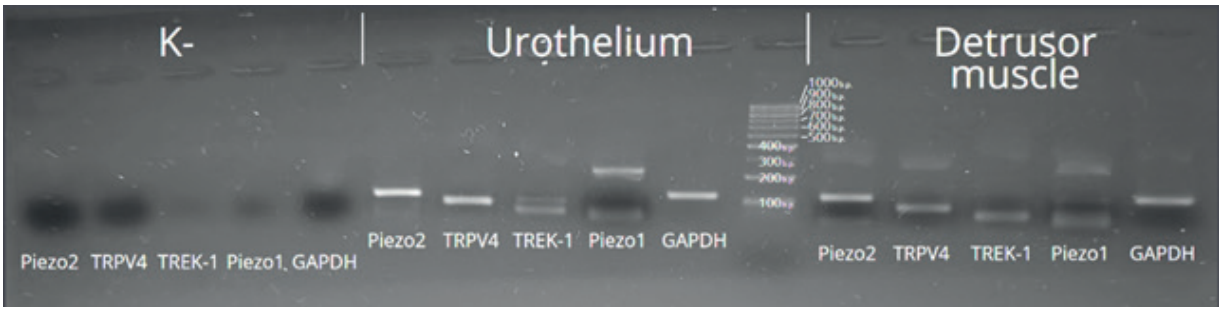

Fig. 1. Piezo1 expression in rat urothelium and detrusor. PCR product from mRNA samples and negative control for all primers (K-). Detrusor expressed Piezo1 channel mRNA as well as TREK-1, TRPV4, and Piezo2. Urothelium shows the same channel expression, GAPDH as positive RT-PCR control; n = 3. Marker shows a 100 b.p. step changes ("Thermo", USA)

The unexpected effect from Yoda1 on DSM cells encouraged us to investigate typical Piezo1 activity, therefore, $[Ca^{2+}]_{in}$ changes on Yoda1 with Ca^{2+}-sensitive fluorescent dye Fluo-4 AM was measured. As a result, 20 µmol/l Yoda1 provoked a slight spike with F/Fo = 1.27 ± 0.18 (or 27±1.8%, n = 12). For comparison, control application of 10 µmol/l cholinomimetic agent CCh (muscarinic acetylcholine receptor (mAChRs) agonist) caused a higher spike with F/Fo = 2.15 ± 1.4 (or 115±1.4% (n = 16) (Fig. 4). From myocytes that proved viability (by CCh stimulation), 12 from 16 were Yoda1-sensitive. For the negative control, cells were pre-treated with fresh Tyrode solution, which did not affect the F level.

DISCUSSION

Research of Ca^{2+} conducted mechanosensitive ion channels in detrusor is essential for understanding mechanisms of mechanosensitive self-regulation of the urinary bladder during infill and urination.

In the present study Yoda1, an agonist of nonselective mechanosensitive ion channel Piezo1 has caused a novel unusual response of DSM cells on whole-cell patch-clamp recordings, i.e. inhibition of outward K^+ currents, which wasn`t described previously. We could not see the activation of the Piezo1 on whole-cell recordings of our DSM cells but instead inhibition of K^+ rest currents. The positive control on ICC-like cells and calcimetry with DSM cells and Yoda1 show normal Piezo1-specific activation. Low expression of *piezo1* mRNA in detrusor is mentioned in literature earlier [17, 18], so this could explain why we don't see currents from Piezo1 activation in whole-cell mode.

Some studies show that inhibition of K^+ current of K^+ ATP-sensitive channels (Kir1.6/

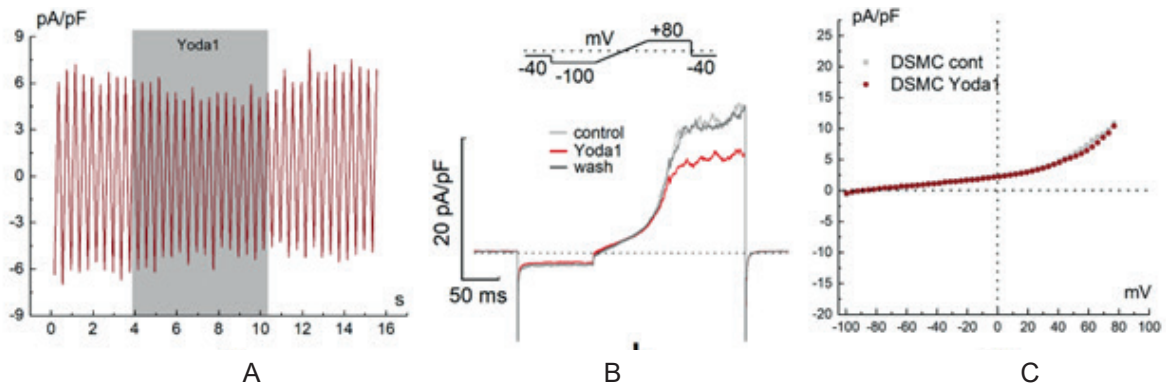

Fig. 2. Yoda1 stimulated inhibition of K^+ currents in DSM cells. a - response to Yoda1 (20 µmol/l) application in DSM cells (n = 11); b - representative recording from DSM cells; c. The I-V curve of Yoda1 stimulated current in DSM cell

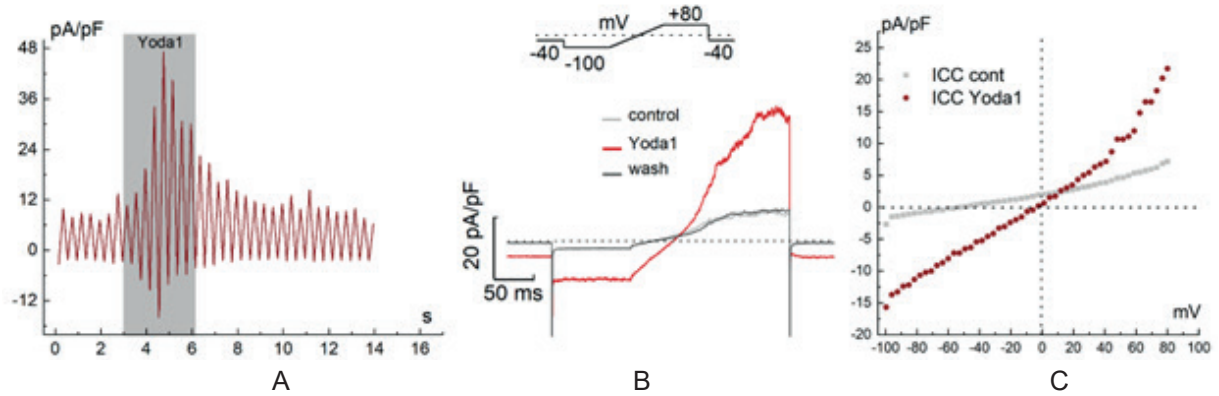

Fig. 3. Yoda1 stimulated currents in ICC cells. a - Piezo1 activation in ICC into the response to Yoda1 (20 μmol/l); b - representative recording from ICC-like cell; c. The I-V curve of Yoda1 stimulated current in ICC-like cell

SUR2B) in column and vascular smooth muscle cells could be regulated by increasing $[Ca^{2+}]_{in}$ [19, 20]. It is known that some K^+ channels are regulated through the phosphorylation/dephosphorylation that is controlled by protein kinases, such as Ca^{2+}/CaM-dependent protein kinase-II (CaMKII) or protein kinase A (PKA), which are regulated by cAMP, Ca-CaM signaling system, and calcium-sensitive phosphatase calcineurin (CaN) [19, 21, 22]. So, Ca^{2+} increase from Piezo1 theoretically could interact with Potassium currents negatively.

CONCLUSIONS

So, in DSM cells selective activation of mechanosensitive, Ca^{2+}-permeable Piezo1 by agonist Yoda1 causes inhibition of the whole-cell rest K^+-currents which could be related to Yoda1 stimulated increase $[Ca^{2+}]_{in}$ and interactions with K^+ channels via some signaling pathways.

This work is dedicated to our friend and colleague, the Hero of Ukraine Dr. Bizhan

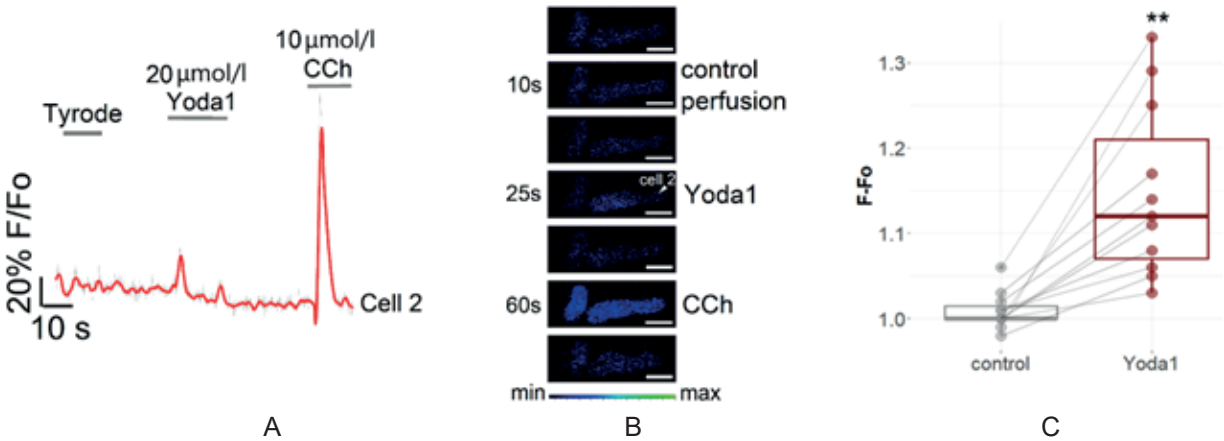

Fig. 4. Yoda1 caused Piezo1-dependent Ca^{2+} influx in DSM cells to a lowered degree: A - representative smoothed graph of F/Fo changes in DSM cells which were stimulated with control perfusion by external Tyrode solution followed by 20 μmol/l Yoda1 and positive control activation by 10 μmol/l CCh; B - Fluo-4 calcimetry timeframe with fluorescent effects from stimulation. The magnitude of changes is represented on a color scale with fake colors which shows fluorescence intensity changes (ΔF), scale bar equal to 10 μm; C. mean fluorescence at peak of control and Yoda1 applications, **P < 0.001 using Welch Two Sample T-test (n = 12)

Sharopov. He was killed at the age of 32 in April 2022 by russians in action in the Kharkiv region after taking part in defence of Kyiv from the first day of russian invasion at Feb-Apr 2022.

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The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

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П'ЄЗО1-ЗАЛЕЖНЕ ПРИГНІЧЕННЯ ФОНОВОГО КАЛІЄВОГО СТРУМУ В ГЛАДЕНЬКОМ'ЯЗОВИХ КЛІТИНАХ СЕЧОВОГО МІХУРА ЩУРІВ

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Гладеньком'язова тканина сечового міхура (детрузор) має автономну механочутливість одночасно з уротеліальним шаром, який є одним з головних сенсорних рецепторів в органі. Piezo1 може відігравати роль локального механорецептора разом з каналом TREK-1 у гладеньком'язових клітинах детрузора сечового міхура (клітини ГМД). Його відносять до так званих «справжніх» механочутливих кальційпровідних іонних каналів, що активується у відповідь на зміни тиску, розтягнення та від селективного агоніста – Yoda1. За допомогою методу patch-clamp та флуоресцентної кальциметрії було показано неочікуваний ефект від Yoda1, а саме інгібування K^+-струмів спокою при позитивних значеннях потенціалу (V_{comm}= + 80 мВ). Функціональна наявність Piezo1 статистично підтверджується методом полімеразної ланцюгової реакції (ПЛР) зі зворотною транскрипцією та індукованим Yoda1 підвищенням внутрішньоклітинної концентрації Ca^{2+} у клітинах ГМД, що візуалізується через чутливий до Ca^{2+} барвник Fluo-4 AM. Тож зроблено висновок, що Piezo1 дійсно функціонує у клітинах ГМД, а також що саме селективна активація каналу його агоністом Yoda1 викликає інгібування калієвих струмів спокою.

Ключові слова: Piezo1; Yoda1; гладеньком'язові клітини; сечовий міхур; калієві струми.

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