Purinergic P2X₇ Receptors Participate in Disturbed Intracellular Calcium Homeostasis in Peripheral Blood Mononuclear Cells of Patients with Chronic Kidney Disease

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Key Words

P2X₇ receptors • Intracellular calcium • Chronic kidney disease • Human lymphocytes

Abstract

Background: P2X₇ receptors intervene with lymphocyte activation and are responsible for multiple processes, including calcium influx. Here, we studied the participation of P2X₇ receptors in disturbed intracellular calcium homeostasis regulation in early-stage chronic kidney disease (CKD). **Methods:** The study involved 20 healthy volunteers and 20 CKD stage 2–3 patients. The free cytosolic calcium concentration ([Ca²⁺]_i) was measured using fluorimetry. The P2X₇ pore function was evaluated by the fluorescent dye ethidium bromide. **Results:** In peripheral blood mononuclear cells (PBMCs) of patients, [Ca²⁺]_i, intracellular calcium stores and the capacitative calcium entry were increased when compared with healthy subjects. The agonist of P2X₇ receptor BzATP caused a sustained increase in [Ca²⁺]_i in both groups, but the effect was smaller in patients. The antagonist at the P2X₇ receptor KN-62 reduced [Ca²⁺]_i in patients, but had no effect in healthy subjects. In patients, the permeability of ethidium bromide through P2X₇ pores, as well as through BzATP-activated and KN-62-inhibited pores, was distinct from permeability in healthy volunteers. **Conclusions:** These

results demonstrate that the calcium signaling pathway in PBMCs of CKD patients is defective already in CKD stage 2–3, and the pore-forming P2X₇ receptors are involved in these pathophysiological processes. Copyright © 2011 S. Karger AG, Basel

Introduction

The ability of cells to perceive and correctly respond to their environment is the basis of development, tissue repair, and immunity as well as of normal tissue homeostasis. Defects in cellular information processing may be responsible for many diseases. Calcium cations (Ca²⁺) are an important ubiquitous messenger, controlling a broad range of cellular processes. In response to adequate stimuli, cytosolic calcium concentration ([Ca²⁺]_i) increases, oscillates and decreases, leading to the activation, modulation and termination of cell function. In non-excitabile cells, the mechanism of regulated Ca²⁺ entry involves a process known as the capacitative Ca²⁺ entry or the store-operated Ca²⁺ entry, in which the depletion of intracellular stores due to inositol trisphosphate (IP₃) or other Ca²⁺-releasing signals activates a signaling pathway leading to the opening of plasma membrane Ca²⁺ channels [1].

Purinergic nucleotides and receptors represent an autocrine/paracrine system that participates in the regulation of cell function. Nucleotides are a ubiquitous family of extracellular signaling molecules that exert different effects through the interaction with plasma membrane receptors named P2 receptors. Based on their molecular structures, intracellular signal transduction mechanisms and pharmacological profiles, the P2 receptors are divided into two major subgroups, P2X and P2Y [2]. One of the P2X subtypes, P2X₇ receptors, are predominantly expressed by cells of hemopoietic origin, where they participate in immune responses, cell proliferation, cell death, bone formation and reabsorption [3]. It has been shown that P2X₇ receptors are also expressed along the nephron and renal vasculature [4, 5]. The P2X₇ receptor is a ligand-gated cation channel with two transmembrane domains and a trimetric structure in the plasma membrane [6]. These receptors can activate cell membrane permeabilization and also has a wide range of downstream signaling pathways, including the mediation of inflammatory responses and modulation of cell turnover. They exist in two functionally distinct states, one of which corresponds to a channel that opens rapidly in response to agonists and passes small inorganic cations such as Ca²⁺. With prolonged agonist application, these receptors form non-specific pores capable of passing larger organic ions up to 900 Da (in lymphocytes they are permeable to molecules up to 320 Da) [7].

Chronic kidney disease (CKD) has been associated with elevated [Ca²⁺]_i [8, 9]. We have previously reported that [Ca²⁺]_i, intracellular calcium reserves and Ca²⁺ influx through the calcium release-activated calcium (CRAC) channels are increased in human PBMCs in early stages of CKD; thus, the balance between calcium entry and its extrusion or buffering within the cells is impaired. Vitamin D₃ treatment decreased elevated [Ca²⁺]_i in CKD patients, but did not affect intracellular calcium reserves or calcium entry through the CRAC channels [10]. This points to the potential role of purinergic P2X₇ receptors in these processes. The aim of this study was therefore to investigate the participation of pore-forming purinergic P2X₇ receptors in intracellular calcium regulation in CKD patients as compared to healthy volunteers.

Subjects and Methods

Subjects

Twenty healthy volunteers (mean age 42 ± 2, range 26–63 years) and 20 non-diabetic patients with stages 2–3 CKD (mean age 50 ± 3, range 28–64 years) were included in the study. All of

Table 1. Patient clinical diagnosis of CKD

Parameters	n
Glomerulonephritis	9
Tubulointerstitial nephritis	4
Hypertensive nephroangiosclerosis	5
Other	2

Table 2. Biochemical parameters in CKD patients

Parameters	Mean values ± SE
Total serum calcium, mmol/l	2.39 ± 0.02
iPTH, pg/ml	56 ± 15
25(OH)D ₃ , ng/ml	24 ± 5
1,25(OH) ₂ D ₃ , pg/ml	29 ± 5
Serum creatinine, μmol/l	138 ± 18
eGFR, ml/s	0.90 ± 0.08

them were screened and followed in outpatient departments of nephrology and internal medicine at the Slovak Medical University. The diagnosis of CKD (table 1) was based on clinical and laboratory examinations as defined by the K/DOQI criteria [11]. The glomerular filtration rate was estimated by the MDRD study formula [12]. Patients with acute impairment of renal function, nephrotic proteinuria, malignancies and derangements in mineral metabolism of non-renal origin were excluded from the study. Concurrent treatments interfering with mineral metabolism (corticosteroids, calcitonin, bisphosphonates, fluorides, calcimimetics, phenytoin, barbiturates) were not allowed. Previous therapy with vitamin D₂/D₃, calcitriol or over-the-counter vitamin D preparations had to be cancelled at least 2 months before enrollment. Hypertension was the most common comorbidity present in all but 1 patient and treated with ACE inhibitors or angiotensin II receptor blockers in 17, diuretics in 14, β-blockers in 9, and calcium channel blockers (amlodipine or felodipine) in 8 cases. Dihydropyridine calcium channel blockers were allowed as they do not interfere with studied parameters and effects in PBMCs [13]. The mean ± SE blood pressure values were 141 ± 11/82 ± 6 mm Hg. Other most often used drugs included statins (in 8 patients) and allopurinol (in 5 patients). Total serum calcium, intact parathyroid hormone (iPTH), 25-hydroxyvitamin D₃ (25(OH)D₃) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) were measured in CKD patients to characterize their calciotropic hormone status. Total serum calcium, iPTH and 1,25(OH)₂D₃ levels were normal in CKD patients stages 2–3, while the mean 25(OH)D₃ concentration was lower than the recommended target of 30 ng/ml, thus indicating vitamin D insufficiency (table 2). The total number of lymphocytes, monocytes and natural killer cells of patients with CKD stages 2–3 was similar to healthy donors and the proportions of T and B cells were not different between both groups (data not shown).

The Ethics Committee of Slovak Medical University approved the study and all participants gave their written informed consent.

Cell Isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation from blood that was withdrawn into heparinized syringes, as described previously [14]. Briefly, samples were diluted 1:1 with RPMI-1640 medium, layered onto an equivalent volume of Lymphocyte Separation Medium LSM-1077, and centrifuged at 700 g for 20 min at 22°C. The resulting PBMC layer was washed in 40 ml RPMI, and resuspended in 10 ml RPMI supplemented with 10% fetal bovine serum. Before loading with a fluorescent probe, the cells were centrifuged at 300 g (10 min at 22°C), the supernatant was removed and the pellet was resuspended in 2 ml aliquots of physiological salt solution containing (in mmol/l) 140 NaCl, 5.4 KCl, 1 CaCl₂, 1 Na₂HPO₄, 0.5 MgCl₂, 5 glucose, and 5 HEPES (pH = 7.4). In Ca²⁺-free physiological solution, 1 mmol/l ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (pH = 7) and 1 mmol/l MgCl₂ was substituted for CaCl₂. In this way, a final concentration of 2 × 10⁶ cells/ml solution was obtained. Our preparation contained lymphocytes (90–94%), monocytes (3–6%), and natural killer cells (the rest), as determined by flow cytometry (Coulter Epics XL, Ireland). The cell viability was quantified using a 0.8% solution of trypan blue and estimated to be 96–98%.

Measurement of [Ca²⁺]_i

The population of 2 × 10⁶ cells/ml was loaded with Fluo-3 acetoxymethylester (Fluo-3 AM) at a final concentration of 2 μmol/l for 40 min at 22°C in a physiological salt solution. After incubation, the cells were centrifuged at 300 g, washed 3 times with a physiological salt solution, and kept at room temperature for 10 min before use. The Fluo-3 fluorescence was measured at 37°C in a Fluorolog 3-11 spectrofluorimeter (Horiba Jobin Yvon Inc., Edison, N.J., USA) with an excitation at 488 nm (bandpass 3 nm) and an emission at 526 nm (bandpass 5 nm). Each experiment was followed by [Ca²⁺]_i calibration to estimate the actual free cytoplasmic calcium concentration from the measured fluorescence signal (F) in each cell population. [Ca²⁺]_i was quantified in nmol/l according to the equation:

$$[Ca^{2+}]_i = K_d \cdot [(F - F_{min}) / (F_{max} - F)]$$

where K_d = 400 nmol/l at 37°C [15]. The maximal fluorescence intensity (F_{max}) was assessed by the addition of Triton X-100 (0.1%) with Ca²⁺ (5 mmol/l), and the minimum fluorescence level (F_{min}) was determined after the addition of 25 mmol/l EGTA (pH = 9). Digitonin (20 μmol/l) was used to answer for minimal compartmentalization [16].

To determine the calcium concentration of thapsigargin (Tg)-sensitive stores, Tg (1 μmol/l), a specific inhibitor of endoplasmic reticulum Ca²⁺-ATPase [17], was used. The experiments were performed in the absence of extracellular calcium in the external medium and calcium released from the cells was chelated with EGTA (pH = 7).

To examine the capacitative calcium entry, 2-aminoethyl-diphenylborate (2APB), an inhibitor of the capacitative calcium entry channels [18], was applied. The action of 2APB (50 μmol/l)

was studied in cells where the capacitative calcium entry was stimulated by Tg (1 μmol/l). 4-[(2S)-2-[(5-isoquinolinesulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl]phenyl isoquinolinesulfonic acid ester (KN-62) (1 μmol/l), a potent non-competitive antagonist of P2X₇ receptors, and 2',3'-O-(4-benzoyl)benzoyl ATP (BzATP) (50 μmol/l), currently the most potent and selective agonist of these receptors, were used to examine the function of cation channel P2X₇ receptors.

Estimation of Ethidium Bromide Entry

BzATP-activated and KN-62-inhibited pore functions were measured using fluorescent indicator ethidium bromide at a final concentration of 30 μmol/l. The transmission and the fluorescence imaging were done by laser scanning confocal microscope LSM 510 META on an Axiovert 200 inverted microscope (Carl Zeiss, Germany). We used a 40×/1.2 W C-Apochromat water-immersion objective and confocal pinhole opening of 1 Airy unit. A 514-nm argon-ion laser line was used as an excitation source and a 545-nm dichroic mirror and 560-nm long-pass emission filter were used for fluorescence detection. Areas of 230 × 230 μm were scanned with a resolution of 1,024 × 1,024 pixels. Fluorescence images were processed in the LSM software as described previously [13], creating ethidium bromide intensity profiles along the broken line, passing through all fluorescing cells in the scan. Intensity of ethidium bromide fluorescence per cell was estimated similarly to the cytometric approach, counting the mean fluorescence intensity of each fluorescing cell, normalized by the total number of cells. The total number of PBMCs in each scan was determined from transmission images, taken simultaneously with fluorescence measurements.

Reagents

Extracellular physiological salt solution contained (in mmol/l): 140 NaCl, 5.4 KCl, 1 CaCl₂, 1 Na₂HPO₄, 0.5 MgCl₂, 5 glucose, and 5 HEPES (pH = 7.4). In Ca²⁺-free physiological solution, 1 mmol/l EGTA (pH = 7) and 1 mmol/l MgCl₂ were substituted for CaCl₂. Tg was procured from Calbiochem (San Diego, Calif., USA) and 2APB, KN-62 and BzATP were obtained from Sigma (St. Louis, Mo., USA). Ethidium bromide (Sigma) was prepared freshly and used at final concentrations 30 μmol/l. Fluo-3 AM was from Molecular Probes (Eugene, Oreg., USA), Lymphocyte Separation Medium LSM-1077 from PAA Laboratories GmbH (Pasching, Austria), and fetal bovine serum and RPMI-1640 medium from Gibco (Grand Island, N.Y., USA). All other chemicals were purchased from Sigma. Stock solutions were prepared in dimethylsulfoxide (Tg, Fluo-3 AM, KN-62) and ethanol (2APB).

Sample Analysis

Total calcium and creatinine (Vitros 250 Analyzer; Johnson & Johnson, Rochester, N.Y., USA) were measured in serum samples. Intact parathormone was determined by immunoradiometric analysis (Immunotech, Marseille, France), 25(OH)D₃ and 1,25(OH)₂D₃ by RIA methods (Immunodiagnostic Systems, Boldon, UK).

The white blood cells and percent of lymphocytes were routinely measured on whole blood samples using the automated hematology analyzer Sysmex KX-21 (TOA Medical Electronics Co., Ltd., Kobe, Japan). The flow cytometric analysis of peripheral blood leukocyte subpopulations was performed on the Cytomics FC 500 using CXP software (both from Beckman Coulter, Ireland).

Fig. 1. Free intracellular calcium concentration ($[Ca^{2+}]_i$), intracellular Tg-sensitive calcium reserves and capacitative calcium entry in PBMCs of healthy subjects and CKD patients. Comparison of $[Ca^{2+}]_i$ in PBMCs of healthy subjects and CKD patients (**a**). Data are means $\pm$ SE (** $p < 0.001$). The intracellular calcium reserves were determined using Tg (1 μ mol/l), a specific inhibitor of endoplasmic reticulum Ca^{2+} -ATPase, in the absence of extracellular calcium, chelated by EGTA (pH 7.0) in the external medium. The

calcium concentration of Tg-sensitive stores of patients with CKD was significantly increased (**b**). Data are means $\pm$ SE (* $p < 0.01$). Typical experiments where CRAC channels were stimulated with Tg (1 μ mol/l) prior to 2APB (50 μ mol/l) application. Representative traces were obtained from a single experiment from PBMCs of a healthy volunteer and CKD patient (**c**). Comparison of capacitative calcium entry in PBMCs of healthy subjects and CKD patients (**d**). Data are means $\pm$ SE (** $p < 0.001$).

Statistical Analysis

Results are expressed as means $\pm$ SE. The Shapiro-Wilk test was used to evaluate sample normality distribution. The statistical significance of differences was tested by independent two-population Student's t test. Spearman's correlations between variables were used as a measure of association. A p value of < 0.01 was considered statistically significant. Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, Ill., USA).

Results

Free Intracellular Calcium, Intracellular Calcium Reserves and the Capacitative Calcium Entry

$[Ca^{2+}]_i$ was estimated to reach 102 ± 1 nmol/l in PBMCs of healthy volunteers while it was substantially

higher in CKD patients (125 ± 1.8 nmol/l; $p < 0.001$) (fig. 1a). To examine the calcium concentration of intracellular Tg-sensitive stores, the effect of Tg (1 μ mol/l) in the absence of extracellular calcium, chelated with EGTA (pH = 7) in the external medium, was investigated. The calcium concentration of Tg-sensitive stores was significantly increased in CKD patients in comparison with healthy volunteers (160 ± 16.9 vs. 113 ± 7.7 nmol/l; $p < 0.01$) (fig. 1b). To examine the capacitative calcium entry, CRAC channels were activated by intracellular Ca^{2+} store depletion using Tg (1 μ mol/l), a specific inhibitor of endoplasmic reticulum Ca^{2+} -ATPase. The inhibitor of capacitative calcium entry, 2APB (50 μ mol/l), was applied during the sustained phase of Tg effect. It evoked a decrease in $[Ca^{2+}]_i$, which represented Ca^{2+} influx

Fig. 2. Effect of BzATP, the most potent agonist of P2X₇ receptors, on intracellular calcium concentration. Comparison of $[Ca^{2+}]_i$ under control conditions and after stimulation with BzATP (50 μ mol/l) in healthy subjects and patients with CKD (** $p < 0.001$; **a**). The differences in $[Ca^{2+}]_i$ after BzATP stimulation in healthy subjects and CKD patients (* $p < 0.01$; **b**). All values are means $\pm$ SE.

Fig. 3. Effect of KN-62, selective antagonist of purinergic P2X₇ receptors, on intracellular calcium concentration. Comparison of $[Ca^{2+}]_i$ under control conditions and after an inhibition with KN-62 (1 μ mol/l) in healthy subjects and patients with CKD (* $p < 0.01$; **a**). The differences in $[Ca^{2+}]_i$ after KN-62 inhibition in healthy subjects and CKD patients (** $p < 0.001$; **b**). Data are means $\pm$ SE.

through CRAC channels (fig. 1c). The capacitative calcium entry was higher in CKD patients than in the healthy subjects (80 ± 5.9 vs. 36 ± 2.7 nmol/l; $p < 0.001$) (fig. 1d).

Calcium Entry through P2X₇ Cation Channels

The most potent agonist of purinergic P2X₇ receptors, BzATP (50 μ mol/l), caused a sustained increase in $[Ca^{2+}]_i$ in both groups (fig. 2a), but the effect was significantly smaller in CKD patients (51 ± 4.9 vs. 29 ± 4.9 nmol/l; $p < 0.01$) (fig. 2b).

Although purinergic receptors did not activate with their agonist, the application of P2X₇ receptor antagonist KN-62 (1 μ mol/l) decreased $[Ca^{2+}]_i$ in CKD patients from 125 ± 1.8 to 114 ± 3.9 nmol/l ($p < 0.01$) (fig. 3a), while in healthy volunteers it had no effect on $[Ca^{2+}]_i$ (fig. 3a, b).

In BzATP-activated cells, KN-62 (1 μ mol/l) decreased calcium influx in both groups (fig. 4a, b). However, the effect of KN-62 was attenuated in PBMCs of CKD patients when compared with healthy volunteers ($p < 0.001$) (fig. 4c). Furthermore, the effect of BzATP on KN-62-inhibited calcium influx in PBMCs was evaluated. In these conditions

Fig. 4. Effect of KN-62 on BzATP-stimulated PBMCs. Typical traces where KN-62 (1 $\mu\text{mol/l}$) inhibited the $[\text{Ca}^{2+}]_i$ rise induced by BzATP (50 $\mu\text{mol/l}$). Representative traces were obtained from a single experiment from PBMCs of a healthy volunteer and CKD patient (a). $[\text{Ca}^{2+}]_i$ in BzATP-activated PBMCs and PBMCs pretreated with BzATP followed by KN-62 application in healthy subjects and CKD patients (b). Data are means $\pm$ SE (* $p < 0.01$, ** $p < 0.001$). Comparison of the effect of KN-62 (1 $\mu\text{mol/l}$) on $[\text{Ca}^{2+}]_i$ in BzATP-activated PBMCs in healthy subjects and patients of CKD (c). Data are means $\pm$ SE (** $p < 0.001$).

the rising calcium influx through P2X_7 channels was found in both groups (fig. 5a, b) and the differences in $[\text{Ca}^{2+}]_i$ between these groups were not measured (fig. 5c).

Pores of P2X_7 Receptors

The permeability of ethidium bromide through P2X_7 pores in PBMCs in the absence or presence BzATP and KN-62, respectively, was examined using separate fluorescence measurements. The ethidium bromide fluorescence intensity of tested cell populations was estimated in control conditions. We observed a significant increase in fluorescence intensity of CKD patients in comparison with healthy volunteers ($p < 0.01$) (fig. 6a). The application of 50 $\mu\text{mol/l}$ BzATP stimulated the entry of ethidium bromide into PBMCs of healthy volunteers, but the ethidium bromide fluorescence was not significantly enhanced in CKD patients. The fluorescence intensity of BzATP-activated cells was comparable in both groups (fig. 6a). The permeability of ethidium bromide through

P2X_7 pores in PBMCs of CKD patients was reduced by 10 min pretreatment with KN-62 (1 $\mu\text{mol/l}$). The cells of healthy subjects did not show the changes in ethidium bromide fluorescence (fig. 6a, b).

Discussion

Different organs and cells closely communicate by means of processes which maintain homeostasis, stimulate adaptation, and induce an appropriate response to injury. This cross-talk includes various systems such as signal transduction pathways, ion flows, receptors, and others. P2X_7 receptor is a bifunctional purinoreceptor that either opens a non-selective cation channel or forms a large, cytolytic pore. ATP leads to the opening of cation channel that permits K^+ efflux out of and Ca^{2+} and Na^+ influx into the cells. This causes a major disturbance to the ionic gradient across the plasma membrane allowing

Fig. 5. Effect of BzATP on KN-62-inhibited PBMCs. Typical experiments where PBMCs were pretreated with KN-62 (1 μ mol/l) prior to the application of BzATP (50 μ mol/l). Representative traces were obtained from a single experiment from PBMCs of a healthy volunteer and CKD patient (**a**). Free cytosolic calcium in the PBMCs inhibited with KN-62 (1 μ mol/l) in healthy subjects and patients with CKD (* $p < 0.01$; **b**). Comparison of BzATP effect on $[Ca^{2+}]_i$ in KN-62-inhibited PBMCs in healthy subjects and patients with CKD (**c**). Data are means $\pm$ SE.

Fig. 6. Ethidium bromide fluorescence intensity of tested cell populations. Ethidium bromide was added to cell suspensions (2×10^6 cells/ml) 30 s before BzATP (50 μ mol/l) or KN-62 (1 μ mol/l) application. The fluorescence images were taken after 10 min exposure to BzATP or KN-62. Comparison of ethidium bromide PBMC permeability under control conditions, after stimulation

with BzATP and inhibition with KN-62 in healthy subjects and CKD patients (* $p < 0.01$; **a**). Comparison of the effects BzATP and KN-62 on ethidium bromide permeability PBMCs of healthy subjects and patients with CKD (* $p < 0.01$; **b**). The intensity of ethidium bromide fluorescence was determined as described in Subjects and Methods. All values are means $\pm$ SE.

calcium influx into the cell, thus triggering several intracellular signaling cascades including mediation of inflammatory responses and modulation of cell turnover. A key factor of P2X₇-dependent cytotoxicity is the massive intracellular Ca²⁺ increase triggered by its activation. Activation of the ligand-gated cation channel after brief exposure of ATP leads to plasma membrane blebbing and apoptosis, and processing rapid release of interleukin-1 β . Prolonged exposure to ATP leads to the formation of a membrane pore which results in cell death by lysis and necrosis [19]. There is an increasing body of evidence implicating P2X₇ receptor in various pathological conditions, such as rheumatoid arthritis, neurological disorders, hypertension, atherosclerosis, and renal dysfunction, etc. [5, 20–22].

Turner et al. [23] showed the distribution of P2X₇ receptor in diseased renal tissue. This study proved the expression of P2X₇ receptors in glomeruli, podocytes, endothelial and mesangial cells and fibroblasts in diabetic and hypertensive rats. In an experimental model of glomerulonephritis, Taylor et al. [24] indicated a significant and possibly key proinflammatory role of the P2X₇ receptor in autoimmune renal injury and provided evidence that P2X₇ antagonists may prevent the development of this type of renal disease. A physiological role of these receptors in the kidney and other tissues is beginning to emerge. So far, no human studies focusing on the evaluation of disturbances in intracellular calcium homeostasis in connection with altered P2X₇ function in renal disease have been published.

The primary aim of our studies is to elucidate the pathophysiological mechanisms of altered intracellular calcium homeostasis in patients with CKD. For this purpose, isolated human peripheral blood mononuclear cells were used as a model of non-excitabile cells. In the present study we have demonstrated that the calcium signaling pathway in these cells is already defective in the early stages of CKD. Purinergic P2X₇ receptors are involved in altered intracellular calcium signaling and the permeation of P2X₇ pores in this disease.

Free intracellular calcium represents a critical signaling mediator in a number of biological systems. Many cellular functions are directly or indirectly regulated by [Ca²⁺]_i. Therefore, [Ca²⁺]_i must be tightly regulated in space, frequency and amplitude because the localization and integrated [Ca²⁺]_i over time can contain specific information. [Ca²⁺]_i is controlled by mechanisms that regulate Ca²⁺ entry from the extracellular space and Ca²⁺ release from intracellular stores, and by the activity of ATP-dependent Ca²⁺ pumps and antiporters that move Ca²⁺

back into stores or out of cells. Chronic renal failure is associated with a significant elevation in [Ca²⁺]_i which is toxic to the cells and may be responsible for a multiple organ dysfunction [8, 9]. Disturbances in intracellular calcium homeostasis in patients with CKD represent a complex process. Previous studies have demonstrated that this elevation is accompanied by the decrease in plasma membrane Ca²⁺-ATPase (PMCA) activity [25, 26], by the enhanced calcium influx [9] and the increase in intracellular calcium reserves [10]. The main mechanism of the lymphocyte [Ca²⁺]_i increase involves CRAC channels. To study potential disturbances associated with this pathway in CKD, CRAC channels were activated by intracellular Ca²⁺ store depletion using Tg, a specific inhibitor of endoplasmic reticulum Ca²⁺-ATPase prior to the application of 2APB, an inhibitor of CRAC channels. We demonstrated that already in early stages of CKD, Ca²⁺ influx through CRAC channels is significantly higher in comparison with healthy volunteers. In lymphocytes, Ca²⁺ influx through these channels is an important regulator of PMCA activity. Bautista and Lewis [27] found an intimate functional association between CRAC channels and PMCA. Taking all these facts into consideration, it can be supposed that one of the reasons of PMCA malfunction in CKD is an altered communication between CRAC channels and PMCA. In addition to CRAC channels, calcium/cationic channels such as L-type and P2X channels are also involved in the increase of [Ca²⁺]_i. The presence of P2X₇ type purinergic receptors was demonstrated in human lymphocytes [22] as well as in other blood cells [3, 28]. Stimulation of P2X₇ receptors induces cell membrane depolarization, an increase in [Ca²⁺]_i and the formation of large cytolytic pores in the cell membrane. In our previous studies, we demonstrated that calcitriol, an active metabolite of vitamin D, prevents Ca²⁺ increase through P2X₇ channels and reduces the membrane permeability through P2X₇ pores [13]. Moreover, vitamin D₃ supplementation in early stages of CKD decreased [Ca²⁺]_i but did not affect intracellular calcium reserves and calcium entry through CRAC channels [10]. These findings point to the potential role of P2X₇ receptors in enhanced [Ca²⁺]_i in this disease. The functionality of these receptors was shown by different assays such as ligand-induced changes in [Ca²⁺]_i and the estimation of ethidium bromide entry. P2X₇ receptors are activated by BzATP, partially stimulated by high extracellular ATP concentrations [29], and selectively inhibited by KN-62 [30]. Calcium influx and the ethidium bromide permeability through BzATP-activated or KN-62-inhibited receptors were examined in PBMCs of CKD stage 2–3 pa-

tients and healthy volunteers. An inhibitor of P2X₇ receptors, KN-62, had no effect in healthy subjects but decreased [Ca²⁺]_i in CKD patients. The effect of BzATP on calcium influx in CKD patients was lower when compared with healthy volunteers. These effects signify that P2X₇ channels are partially open in CKD patients. Further experiments presented in this study demonstrate that the function of P2X₇ channels is altered in CKD. In addition, the permeability of ethidium bromide through plasma membrane pores was higher in PBMCs of CKD patients. The comparison of ethidium bromide fluorescence intensity of the tested cells after BzATP and KN-62 application pointed to altered P2X₇ pore function in CKD. No association between age and evaluated effects was found in either healthy volunteers or CKD patients in any of the experiments. Taken together, the distinct function of pore-forming purinergic P2X₇ receptors may contribute to the complex disturbances in intracellular calcium homeostasis in CKD. At least 250 polymorphic forms of the P2X₇ receptor have been identified [31]. This multiplicity is associated with the extended length of the

C-terminal region of this receptor relative to other P2X receptors [32]. Both gain-of-function [33] and loss-of-function single nucleotide polymorphisms have been reported [34, 35]. Besides an altered function of P2X₇ receptors, their expression can be changed, too. Recent studies showed that P2X₇ receptors are differentially expressed in the cells under physiological and pathological states [20, 36].

In conclusion, our findings indicate that the balance between calcium entry and its extrusion or buffering within the cells is already impaired in the early stage of CKD. We have provided the first evidence that P2X₇ receptors participate in this altered intracellular calcium homeostasis in CKD patients.

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