The Effect of Vitamin D₃ Supplementation on Intracellular Calcium and Plasma Membrane Calcium ATPase Activity in Early Stages of Chronic Kidney Disease

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Summary

Chronic kidney disease (CKD) is associated with increased concentration of intracellular calcium, which is pathological and may lead to irreversible damage of cell functions and structures. The aim of our study was to investigate the impact of 6 months vitamin D₃ supplementation (14 000 IU/week) on free cytosolic calcium concentration ($[Ca^{2+}]_i$) and on the plasma membrane calcium ATPase (PMCA) activity of patients with CKD stage 2-3. PMCA activity of patients was also compared to that of healthy volunteers. Vitamin D₃ supplementation of CKD patients resulted in the decrease of $[Ca^{2+}]_i$ (119.79±5.87 nmol/l vs. 105.36± 3.59 nmol/l, n=14, P<0.001), whereas PMCA activity of CKD patients (38.75±22.89 nmol P_i/mg/h) remained unchanged after vitamin D₃ supplementation (40.96±17.74 nmol P_i/mg/h, n=14). PMCA activity of early stage CKD patients before supplementation of vitamin D₃, was reduced by 34 % (42.01±20.64 nmol P_i/mg/h) in comparison to healthy volunteers (63.68±20.32 nmol P_i/mg/h, n=28, P<0.001). These results indicate that vitamin D₃ supplementation had a lowering effect on $[Ca^{2+}]_i$ and negligible effect on PMCA activity in CKD patients.

Key words

Calcium-ATPase • Chronic kidney disease • Calcium • Vitamin D₃

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Introduction

Chronic kidney disease (CKD) is defined as a progressive loss of renal function over time, with increasing prevalence of around 10-13 % (Coresh *et al.* 2007, Zoccali *et al.* 2010). It is well known that, among other complications, CKD leads to calcium homeostasis disturbances and so the free cytosolic calcium concentration ($[Ca^{2+}]_i$) in CKD patients is increased (Gafter *et al.* 1990, Zidek *et al.* 1992). Increased $[Ca^{2+}]_i$ in red blood cells (RBCs) results in the decreased cell flexibility, elevated osmotic fragility and, finally hemolysis (Spieker *et al.* 1993). The $[Ca^{2+}]_i$ is precisely controlled in healthy cells. The extracellular ionized calcium concentration is maintained at about 1.2 mmol/l and $[Ca^{2+}]_i$ is much lower (about 10^4 times), typically between 50 and 100 nmol/l. Even minor changes in the permeability of the plasma membrane to Ca^{2+} will produce significant $[Ca^{2+}]_i$ fluctuations (Bronner 2001). This serves the regulation of cellular processes, however long-term increase in Ca^{2+} is pathological and may lead to irreversible damage of cell functions and structures. The maintenance of such a high concentration gradient is possible only due to a finely tuned cooperation between many regulating systems present in cell membranes and organelles. Calcium enters the cell *via* various types of plasma membrane channels (like voltage-gated, stores-gated or receptor-gated channel) and is transported out of the cell by the Ca^{2+}-ATPase (active transport) or the sodium-calcium exchanger. Also, mechanisms of cellular compartments (endoplasmic reticulum and mitochondria)

participate in maintaining calcium homeostasis. Disturbances at any level of this regulation may lead to failure of calcium homeostasis and accumulation of intracellular calcium (Carafoli 1987).

Two basic groups of calcium pumps have been identified in cellular membranes: plasma membrane Ca^{2+}-transport ATPase (PMCA) and Ca^{2+}-transport ATPase (SERCA) within sarco/endoplasmic reticulum. PMCA is a transporting protein, localized in plasma membrane of all eukaryotes belonging to the P-type of primary ion transport ATPases subtype IIB and is characterized by production of aspartyl phosphate intermediate in the reaction cycle (Strehler and Zacharias 2001). During transmission of one calcium ion, the ATP is hydrolyzed into ADP and inorganic phosphate. PMCA can be one of the mechanisms responsible for decrease in $[Ca^{2+}]_i$ in CKD patients. Decreased PMCA activity was reported in patients undergoing dialysis, or patients in terminal stage (4-5) renal failure (Gafter *et al.* 1990, Zidek *et al.* 1992), but only few studies concentrate their interest on early stage of CKD. PMCA activity was showed significantly decreased (about 50 %) in children with CKD in the early stages (2-4) (Polak-Jonkisz *et al.* 2007). But information is missing for adult patients in the early stages of CKD.

Vitamin D plays a crucial role in the regulation of calcium-phosphate homeostasis. The vitamin D deficiency is common in early stages of CKD. Therefore, K/DOQI guidelines recommend supplementation with vitamin D (ergocalciferol or cholecalciferol) (K/DOQI 2003). Only few studies were focused on the effect of vitamin D_3 supplementation on the cellular level and cellular calcium homeostasis in these patients. Therefore, in our study we examined the effect of vitamin D_3 supplementation on the $[Ca^{2+}]_i$ and on the PMCA activity to get more complex insight into influence of the vitamin D_3 on cellular mechanisms in CKD patients.

Methods

Patients

Twenty-eight patients with stages 2-3 of chronic kidney disease (CKD) and the same number of gender-matched healthy volunteers with normal hematological and biochemical values were included in this study. CKD was defined as the presence of kidney damage and/or decrease in glomerular filtration rate and the diagnosis was based on clinical and laboratory examinations as defined by the K/DOQI criteria (K/DOQI 2003). Mean value of age was 59±15 years (mean ± SD) for CKD

patients and 53±14 years for healthy volunteers. All patients were screened and followed up in outpatient departments of nephrology and internal medicine at the Slovak Medical University. The retrieval was realized during time period of January 2011 – October 2013. Patients with acute impairment of renal function, nephrotic-range proteinuria, malignancies and derangements in mineral metabolism of non-renal origin were excluded from the study. Concurrent treatments interfering with mineral metabolism (corticosteroids, calcitonine, bisphosphates, fluorides, calcimimetics, phenytoin, and barbiturates) were not allowed. Fourteen of these patients were exposed to 6 months native vitamin D_3 (cholecalciferol) supplementation at the dose of 14 000 IU/week (Vigantol, Merck KGaA, Germany). After this 6 months lasting vitamin D_3 supplementation the $[Ca^{2+}]_i$ and PMCA activity were measured. The Ethics Committee of Slovak Medical University approved the study and all participants gave their written informed consent.

Sample analyses

Blood samples were extracted by venipuncture from the arm and were collected into tubes containing heparin as an anticoagulant. All measurements were processed on the same day. Plasma was removed and used for the measurement of serum calcium (Vitros 250 Analyzer, Johnson & Johnson, Rochester, NY, USA) and $25(OH)D_3$ by RIA methods (Immunodiagnostic Systems, Boldon, UK). Human peripheral blood mononuclear cells (PBMCs) were used to measure $[Ca^{2+}]_i$ and plasma membrane of red blood cells (RBCs) were used for measurement of PMCA activity. RBCs are the best model to study exclusively the PMCA activity since they avoid the contribution of other intracellular structures besides plasma membrane. It has been demonstrated that mature human erythrocytes lack voltage- and ligand-dependent calcium channels, Na-Ca exchanger and calcium pumps (Stokes and Green 2003).

Human peripheral blood mononuclear cells isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation, as described previously (Lajdova *et al.* 2004). Briefly, samples were diluted 1:1 with RPMI-1640 medium and layered onto an equivalent volume of Lymphocyte Separation Medium LSM-1077. After centrifugation the resulting PBMCs layer was isolated. Before loading with a fluorescent probe the pellet was re-suspended in 2 ml

aliquots of physiological salt solution (for exact composition see *Reagents*). In this way, a final concentration of 2×10^6 cells/ml solution was obtained.

Red blood cell membrane isolation (white ghosts)

Erythrocyte membrane was obtained by hemolytic fragmentation in hypotonic media using standard method Hanahan&Ekholm (Hanahan and Ekholm 1974) modified in our laboratory to achieve a higher quality of the ghosts. RBCs from previous isolation were washed with PBS (20 mmol/l phosphate, 150 mmol/l NaCl, pH 7.4) and centrifuged at 1270 g for 20 min at 4 °C. The supernatant was removed and the procedure was repeated one more time. The sediment was then diluted in ratio 1:5 with TRIS (2-Amino-2-hydroxymethyl-propane-1,3-diol) medium (20 mmol/l, pH 7.4) and centrifuged at 7700 g for 35 min at 4 ºC. This step was repeated twice for each TRIS medium with decreasing concentration (20 mmol/l, 10 mmol/l, 5 mmol/l). In this way white ghosts were achieved.

Measurement of [Ca²⁺]ᵢ

Free cytosolic calcium concentration $[Ca^{2+}]_i$ was measured using the fluorescent probe Fluo 3. The Fluo-3 fluorescence was measured at 37 °C in Fluorolog 3-11 spectrofluorimeter (HORIBA Jobin Yvon Inc., Edison, NJ, USA) with an excitation at 488 nm (bandpass 3 nm) and emission at 526 nm (bandpass 5 nm). Each experiment was followed by $[Ca^{2+}]_i$ calibration to estimate the actual free cytosolic calcium concentration from the measured fluorescence signal (F) in each cell population. $[Ca^{2+}]_i$ was quantified in nmol/l according to the following equation:

$$[Ca^{2+}]_i = K_d \cdot \left[\left(F - F_{\min} \right) / \left(F_{\max} - F \right) \right], \qquad (1)$$

where K_d = 400 nmol/l at 37 °C (Tsien 1988). The maximal fluorescence intensity ($F_{\max}$) was assessed by the addition of Triton X-100 (0.1 %) with Ca^{2+} (5 mmol/l), and the minimum fluorescence level ($F_{\min}$) was determined after the addition of 25 mmol/l EGTA (ethylene glycol tetraacetic acid) (pH 9).

PMCA activity measurement

RBCs membrane suspension was added to working medium containing: 100 mmol/l TRIS, 80 mmol/l KCl, 3 mmol/l MgCl₂, 0.2 mmol/l EDTA (ethylenediaminetetraacetic acid), 1 mmol/l ouabain, pH 7.4 in the presence or absence of 5 mmol/l CaCl₂. The reaction was started by addition of 5 mmol/l ATP and the sample was incubated 60 min at 37 °C. After incubation the reaction was stopped by addition of 15 % TCA (trichloroacetic acid). The amount of inorganic phosphate was detected by phosphate colorimetric assay kit and the chromogenic complex was determined on UV–VIS spectrophotometer Shimadzu UV-1700 (Shimadzu Corp., Japan). The estimated PMCA activity was calculated as the difference between nmol of inorganic phosphate arose by enzyme incubated in the presence or without CaCl₂ and was expressed as nmol of Pᵢ/mg membrane protein/h. The protein content of isolated membrane (ghosts) was assessed by the method of Lowry *et al.* (1951), with the use of Folin reagent and calibration with BSA (bovine serum albumin).

Reagents

The extracellular physiological salt solution containing 140 mmol/l NaCl, 5.4 mmol/l KCl, 1 mmol/l CaCl₂, 1 mmol/l Na₂HPO₄, 0.5 mmol/l MgCl₂, 5 mmol/l glucose, and 5 mmol/l HEPES, (pH 7.4) was used. Fluo-3 acetoxymethylester (Fluo-3-AM) was obtained from Molecular Probes (Eugene, OR, USA), RPMI-1640 medium from GIBCO (Grand Island, NY, USA), Lymphocyte Separation Medium LSM-1077 from PAA Laboratories GmbH (Pasching, Austria). Phosphate colorimetric assay kit was from Biovision (Hayward, CA, USA). All other reagents were from Sigma-Aldrich (St Louis, MO, USA).

Statistical analyses

Data were tested for normal distribution using Shapiro-Wilk W test. Comparison of variances was done using two sided F test. The statistical significance of differences was tested for independent 2-population by Unpaired t test and for paired data by Paired t test or by Wilcoxon's signed ranks test. Calculation, graphical and statistical analysis were performed in Microsoft Excel 2003 and StatsDirect software.

Results

Effect of vitamin D₃ supplementation

Total serum calcium was in normal range and did not change significantly. 25(OH)D₃ was below normal range, but after vitamin D₃ supplementation increased significantly and stayed in normal range (Table 1).

**Table 1.** Total serum calcium and 25(OH)D₃ for CKD patients before and after vitamin D₃ supplementation.

	Before vit. D$_3$	**After vit. D$_3$**
Total serum calcium (mmol/l)	2.27 ± 0.10	2.34 ± 0.08
25(OH)D$_3$ (ng/ml)	18.28 ± 7.72	35.29 ± 8.85[*]

Values are given as mean ± SD (n=14, * P<0.05).

Free intracellular calcium was monitored using the fluorescent probe Fluo 3 AM and its concentration $[Ca^{2+}]_i$ was calculated from the fluorescence signal according to equation 1. $[Ca^{2+}]_i$ was measured on PBMC. Patients were then subjected to 6 months of vitamin D₃ supplementation (14 000 IU/week). Increased $[Ca^{2+}]_i$ (119.79±5.87 nmol/l, mean ± SD) of CKD patients was significantly (P<0.001) reduced after vitamin D₃ supplementation to the value of 105.36±3.59 nmol/l (Fig. 1).
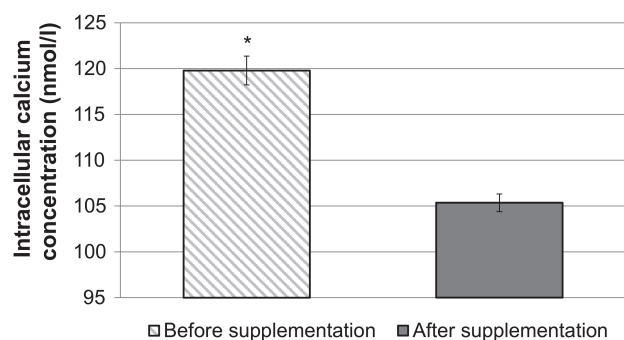
Fig. 1. Comparison of the mean values of free cytosolic calcium concentration (expressed in nmol/l) of PBMC in early stage CKD patients before and after 6 months vitamin D₃ supplementation (* P<0.001, n=14). Data are given as mean ± SEM.

We also investigated the influence of vitamin D₃ supplementation on PMCA activity. Experiments were carried out on RBCs ghosts in medium with and without calcium ions. PMCA activity was determined by colorimetric method which detects inorganic phosphate. Quantity of inorganic phosphate was adjusted to the protein content of isolated membranes and PMCA activity was expressed in nmol of inorganic phosphate per mg of protein in RBCs membrane per hour. Vitamin D₃ supplementation had no effect on PMCA activity (38.75±22.89 nmol P$_i$/mg/h vs. 40.96±17.74 nmol P$_i$/mg/h, n=14) (Fig. 2).
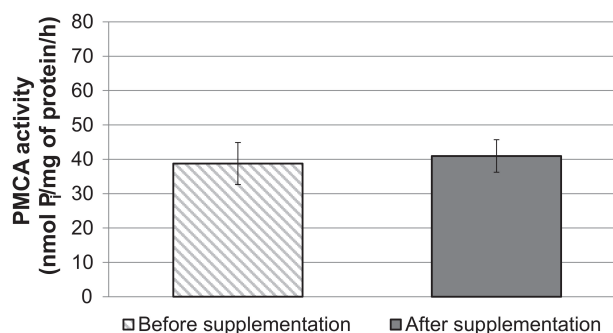
Fig. 2. Comparison of the mean values of PMCA activity of RBC membrane (expressed in nmol of inorganic phosphate (P$_i$) produced per mg of plasma membrane protein per hour) in early stage CKD patients before and after 6 months vitamin D₃ supplementation (n=14). Data are given as mean ± SEM.
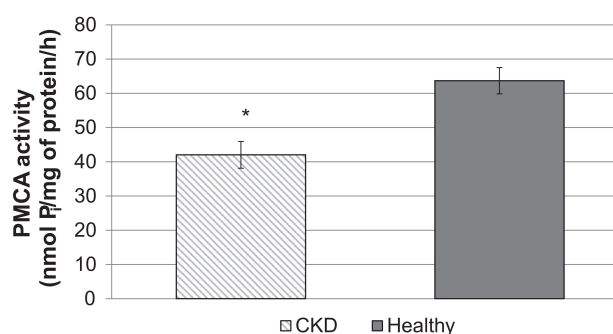
Fig. 3. Comparison of the mean values of PMCA activity of RBCs membrane (expressed in nmol of inorganic phosphate (P$_i$) produced per mg of plasma membrane protein per hour) in early stage CKD patients and healthy donors (* P<0.001, n=28). Data are given as mean ± SEM.
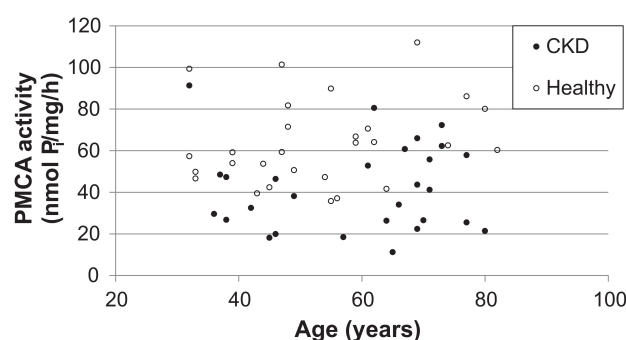
Fig. 4. The dependence of PMCA activity on age of CKD patients (•) and healthy donors (○) (n=28). No significant correlation was detected.

Comparison of PMCA activity in CKD patients and healthy subjects

The PMCA activity was 42.01±20.64 nmol Pi/mg/h for CKD patients (stage 2-3) vs. 63.68±20.32 nmol Pi/mg/h (mean ± SD) for healthy population. These results showed that PMCA activity in patients was significantly (P<0.001) reduced by 34 % as compared to

healthy volunteers (Fig. 3). We also tested a possible correlation between PMCA activity and the age of subjects in both groups. Our findings indicated that there are no correlations between PMCA activity and the age of involved subjects (Fig. 4).

Discussion

In our study, we have shown that after 6 months vitamin D$_3$ supplementation (14 000 IU/week) of CKD patients in stage 2-3 the total serum calcium concentration did not change and remained in normal range. Nevertheless, we have provided evidence that increased [Ca^{2+}]$_i$ (120 (116-123) nmol/l) in CKD patients was significantly decreased after vitamin D$_3$ supplementation and reached the value of 105 (103-107) nmol/l (Fig. 1), which is comparable to those reported for healthy volunteers 102 (99-103) nmol/l (mean (95% CI)) (Lajdova *et al.* 2009). It is not yet fully understood by which cellular mechanisms vitamin D$_3$ can cause the reduction of [Ca^{2+}]$_i$. The calcium homeostasis in healthy cells is strictly regulated by mechanisms of plasma membrane which affect the calcium entry into the cell (such as voltage-gated, store gated or receptor-gated channels), mechanisms of plasma membrane which allow the output of calcium from the cell (such as the sodium-calcium exchanger or PMCA) and also the mechanisms affecting the storage of calcium in intracellular compartments (endoplasmic reticulum, mitochondria) (Bronner 2001).

PMCA may be one of the possible mechanisms, responsible for vitamin D$_3$ influence on changes of [Ca^{2+}]$_i$, that has not yet been studied. In this contribution, we found out that 6 months vitamin D$_3$ supplementation (14 000 IU/week) in early stage CKD patients did not affect the change in PMCA activity (Fig. 2). The similar results have been published in *ex vivo* experiments on animals and on cell lines (Halloran *et al.* 1980), although in animal models a significant increase of the PMCA gene expression was observed (Cai *et al.* 1993, Horst *et al.* 2000). In the work of Polak-Jonskisz *et al.* (2007), the authors suggested that the change in PMCA activity may be due to an inhibitor present in the blood of uremic patients, which is removed from the organism during hemodialysis. The above hypothesis is supported by the fact that PMCA activity increases significantly after dialysis and it becomes comparable to that observed in healthy subjects (Gafter *et al.* 1989). The presence of such an inhibitor is also supported by the studies of Lindner *et al.* (1992).

Moreover, our results confirmed that PMCA

activity of adult patients already in early stages of CKD is significantly reduced (by 34 %) against healthy volunteers (Fig. 3), and thus it may be one of the mechanisms involved in the increased [Ca^{2+}]$_i$ in CKD patients. This assumption has been thus supported by studies in patients with end-stage CKD, where the PMCA activity was half as high compared to that in healthy volunteers (Gafter *et al.* 1989, Zidek *et al.* 1992) and a significant reduction (about 50 %) for children in stages 2-4 (Polak-Jonkisz *et al.* 2007). These results indicate that PMCA activity responsible for the calcium efflux begins to decrease in the early stages of the disease and is getting worse with the disease progression. Now it is obvious that process of decreasing [Ca^{2+}]$_i$ is multi-component. It has been demonstrated that in patients with end-stage renal failure, the calcium influx into erythrocytes is increased, most likely due to elevated erythrocyte membrane permeability in uremic conditions (Gafter *et al.* 1989). In non-excitable cells, the store-operated channels are the major Ca^{2+} entry pathway, in which the emptying of intracellular Ca^{2+} stores activates Ca^{2+} influx through a calcium release activated calcium (CRAC) channels. It also had been reported, that Ca^{2+} influx through CRAC channels are increased in human peripheral blood mononuclear cells of early stage CKD patients (Lajdova *et al.* 2009). There was also increased permeability and expression of P2X$_7$ receptors (Kaderjakova *et al.* 2012).

Our experiment did not find any correlation between PMCA activity and age (Fig. 4). Results are consistent with other authors who rejected dependence of Mg^{2+}-ATPase, Na$^+$/K$^+$-ATPase or Ca^{2+}-ATPase activity on the age, gender or state of menstrual cycle (Reinila *et al.* 1982, Davis *et al.* 1987). ATPase activity varies just in children, in newborns and children up to seven years the value of this activity is higher than in adults and reached the maximum between 8-13 years (Friederichs and Wurz 1990).

In summary, our results have proved that vitamin D$_3$ supplementation in early stage CKD patients did not alter total serum calcium, but was capable of decreasing the free cytosolic calcium concentration [Ca^{2+}]$_i$ to levels observed in healthy volunteers. PMCA activity was unaffected after vitamin D$_3$ supplementation, whereas PMCA activity was lowered in comparison to healthy volunteers. Probably other cellular mechanisms are involved in alterations of [Ca^{2+}]$_i$. Finally, we reject any correlation between PMCA activity and age of the subjects.

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