Intracellular calcium homeostasis in patients with early stages of chronic kidney disease: effects of vitamin D₃ supplementation

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Abstract

Background. Chronic renal failure has been referred to as a state of cellular calcium toxicity. The aim of this study was to investigate the status of free cytosolic calcium ([Ca²⁺]), intracellular calcium reserves and the capacitative calcium entry in peripheral blood mononuclear cells (PBMCs) of early-stage chronic kidney disease (CKD) patients, and to determine the effect of vitamin D₃ supplementation on these parameters.

Methods. The study involved 44 patients with CKD stages 2–3; 27 of them were treated with cholecalciferol (5000 IU/week) for 12 months. [Ca²⁺], was measured using Fluo-3 AM fluorimetry. Intracellular calcium reserves were emptied by the application of thapsigargin (Tg), a specific inhibitor of endoplasmic reticulum Ca²⁺-ATPase. 2-Aminoethyl-diphenyl borate (2APB) was used to examine the capacitative calcium entry.

Results. [Ca²⁺], of CKD patients was substantially higher in comparison with healthy subjects: 123 (115–127) versus 102 (98–103) nmol/l, P < 0.001. The calcium concentration of Tg-sensitive stores and the capacitative calcium entry were also significantly increased in CKD patients. After the 12-month vitamin D₃ supplementation, there was a marked decrease in [Ca²⁺], [105 (103–112) nmol/l, P < 0.001 versus baseline], independently of the increase in 25(OH)D₃ or the decrease in PTH levels. No significant changes in intracellular calcium reserves and the capacitative calcium entry were found.

Conclusions. Our results demonstrate that (1) [Ca²⁺], intracellular calcium stores and the capacitative calcium entry were significantly increased already in early stages of CKD; (2) long-term vitamin D₃ supplementation normalized [Ca²⁺], without any effect on intracellular calcium reserves or the capacitative calcium entry.

Keywords: chronic kidney disease; fluorescence; intracellular calcium; vitamin D₃

Introduction

An increased cytosolic calcium concentration ([Ca²⁺]) is used as a key signalling messenger in the initialization of a wide range of cellular events. Ca²⁺ signals control many cellular functions ranging from short-term responses such as contraction and secretion to longer term regulation of cell growth and proliferation [1,2]. On the other hand, Ca²⁺ signal may induce apoptosis or less specific necrosis under special conditions. Calcium enters the cells by any of the general classes of channels, including voltage-operated channels, second messenger-operated channels, store-operated channels and receptor-operated channels. In non-excitable cells, the major Ca²⁺ entry pathway is the store-operated one, in which the emptying of intracellular Ca²⁺ stores activates Ca²⁺ influx (capacitative calcium entry). The increase in [Ca²⁺], has been associated with cellular dysfunctions in a variety of conditions such as diabetes mellitus, hypertension, hyperparathyroidism and chronic renal failure [3–5]. Dysregulation of Ca²⁺ homeostasis involving the endoplasmic reticulum and store-operated calcium channels has been manifested in neurodegenerative disorders such as Alzheimer’s disease [6], in patients with immunodeficiency [7,8], acute pancreatitis [9] and polycystic kidney disease [10–12].

Vitamin D plays a pivotal role in the regulation of calcium-phosphate homeostasis. Vitamin D insufficiency/deficiency is a significant risk factor for the development of specific chronic diseases [13]. In chronic kidney disease (CKD), vitamin D deficiency with low 25(OH)D₃ levels is highly prevalent and associated with secondary hyperparathyroidism, leading to serious mineral and bone disorders [14]. Therefore, the National Kidney Foundation (K/DOQI) guidelines recommend vitamin D (ergocalciferol or cholecalciferol) supplementation already in patients with stage 3 CKD [15]. Treatment with vitamin D sterols (calcitriol or synthetic vitamin D analogues) is well established in CKD patients. So far, studies targeting at the effects of plain vitamin D₃ supplementation on cellular...
The aim of the present study was to determine free cytosolic calcium concentration ([Ca$^{2+}$]i), concentration of intracellular calcium reserves and the capacitative calcium entry into peripheral blood mononuclear cells (PBMCs), and to examine the effect of vitamin D$_3$ supplementation on these parameters in early-stage CKD patients.

**Subjects and methods**

**Subjects and analyses**

Forty-four patients with stages 2–3 CKD (median age 62, range 19–77 years) were included in the study. All of them were screened and followed up in outpatient departments of nephrology and internal medicine at the Slovak Medical University. The enrollment was realized during the time period of April 2004–June 2005. CKD was defined as the presence of kidney damage and/or decreased glomerular filtration rate according to the K/DOQI criteria [15]. The diagnosis of nephropathy was based on clinical and laboratory examinations (Table 1). Those 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, calcitonine, bisphosphonates, fluorides, calcimimetics, phenytoin, barbiturates) were not allowed. Previous therapy with vitamin D$_2$-D$_3$, calcitriol or over-the-counter vitamin D preparations had to be cancelled at least 2 months before enrolment. Total serum calcium, ionized calcium, iPTH, 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ levels, as well as [Ca$^{2+}$]$_i$, were measured at baseline. Then, the patients were supplemented with vitamin D$_3$ (cholecalciferol) 5000 IU/week for 12 months; the dose (approximately 770 IU/day) was chosen as a common supplementary dose for the pre-treatment period. The diagnosis of nephropathy was based on clinical and laboratory examinations (Table 1). Those 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, calcitonine, bisphosphonates, fluorides, calcimimetics, phenytoin, barbiturates) were not allowed. Previous therapy with vitamin D$_2$-D$_3$, calcitriol or over-the-counter vitamin D treatments had to be cancelled at least 2 months before enrolment. Total serum calcium, ionized calcium, iPTH, 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ levels, as well as [Ca$^{2+}$]$_i$, were measured at baseline. Then, the patients were supplemented with vitamin D$_3$ (cholecalciferol) 5000 IU/week for 12 months; the dose (approximately 770 IU/day) was chosen as a common supplementary dose for the prevention of vitamin D deficiency in the general population. Laboratory examinations were repeated after 4 and 12 months of treatment. Control values of [Ca$^{2+}$]$_i$ were determined in 70 healthy volunteers (median age 34, range 18–45 years). The calcium concentrations of thapsigargin (Tg)-sensitive stores were determined in 15 healthy subjects and 15 CKD patients at baseline and after a 12-month supplementation. The capacitative calcium entry was measured in 10 subjects from each group. The cell viability was quantified using a 0.8% solution of trypan blue and estimated to be 96–98%.

**Table 1. Patient clinical diagnoses of CKD**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulointerstitial nephritis</td>
<td>13</td>
</tr>
<tr>
<td>Hypertensive nephroangiosclerosis</td>
<td>5</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>8</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>2</td>
</tr>
<tr>
<td>Atherosclerotic/ischemic nephropathy</td>
<td>3</td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td>3</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
</tr>
<tr>
<td>Combination of ≥2 diagnoses*</td>
<td>6</td>
</tr>
</tbody>
</table>

*In 5/6 combined diagnoses, diabetic nephropathy was supposed.

**Cell isolation**

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation from blood that was withdrawn into heparinized syringes, as previously described [16]. Briefly, the samples were diluted 1:1 with RPMI-1640 medium, layered onto an equivalent volume of Histopaque-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 (FBS). 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 a physiological salt solution containing (in mmol/l) 140 NaCl, 5.4 KCl, 1 CaCl$_2$, 1 Na$_2$HPO$_4$, 0.5 MgCl$_2$, 5 glucose and 5 HEPES (pH = 7.4). In Ca$^{2+}$-free physiological solution, 1 mmol/l ethylene glycol-bis(B-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (EGTA) (pH = 7) and 1 mmol/l MgCl$_2$ was substituted for CaCl$_2$. In this way, a final concentration of 2 × 10$^6$ cells/ml solution was obtained. The cell viability was quantified using a 0.8% solution of trypan blue and estimated to be 96–98%.

**Measurement of [Ca$^{2+}$]$_i$**

The population of 2 × 10$^6$ cells/ml was loaded with 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 three 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 Fluorolog 3–11 spectrofluorometer (HORIBA Jobin Yvon Inc., Edison, NJ, USA) with an excitation at 488 nm (bandpass 3 nm) and an emission at 526 nm (bandpass 5 nm).

Each experiment was followed by [Ca$^{2+}$]$_i$ calibration to estimate the actual free cytoplasmic calcium concentration from the measured fluorescence signal (F) in each cell population. [Ca$^{2+}$]$_i$ was quantified in mmol/l according to the following equation:

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

where $K_d = 400$ mmol/l at 37°C [17]. The maximal fluorescence intensity ($F_{\text{max}}$) was assessed by the addition of Triton X-100 (0.1%) with Ca$^{2+}$ (5 mmol/l), and the minimum fluorescence level ($F_{\text{min}}$) was determined after the addition of 25 mmol/l EGTA ($\phi = 9$). Digitonin (20 µmol/l) was used to answer for minimal compartmentalization [18].

To determine the calcium concentration of Tg-sensitive stores, Tg (1 µmol/l), a specific inhibitor of endoplasmic reticulum Ca$^{2+}$-ATPase [19], was used. The experiments were performed in the absence of extracellular calcium in the external medium, and calcium release from the cells was chelated with EGTA ($\phi = 7$).

To examine the capacitative calcium entry, 2-aminoethyl-diphenylborate (2APB), an inhibitor of the capacitative calcium entry channels [20], 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).

**Reagents**

An extracellular physiological salt solution containing (in mmol/l) 140 NaCl, 5.4 KCl, 1 CaCl$_2$, 1 Na$_2$HPO$_4$, 0.5 MgCl$_2$, 5 glucose and 5 HEPES, (pH = 7.4) was used. In a Ca$^{2+}$-free physiological solution, 1 mmol/l ethylene glycol-bis(B-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (EGTA) (pH = 7) and 1 mmol/l MgCl$_2$ were substituted for CaCl$_2$. Thapsigargin (Tg) was procured from Calbiochem (San Diego, CA, USA), and 2-aminoethyldiphenylborate (2APB) was obtained from Sigma (St Louis, MO, USA). Fluo-3 acetyoxymethyl ester (Fluo-3 AM) was obtained from Molecular Probes (Eugene, OR, USA), and fetal bovine serum (FBS) and RPMI-1640 medium from Gibco (Grand Island, NY, USA). Stock solutions were prepared in dimethylsulfoxide (Tg, Fluo-3 AM) and ethanol (2APB). All other chemicals were purchased from Sigma.

**Sample analyses**

Calcium (Vitros 250 Analyzer, Johnson & Johnson, Rochester, NY, USA) and ionized calcium (iCa) (Nova 8 CRT Analyzer, Nova Biomedical, Valtham, MA, USA) were measured in serum samples. Intact parathormone (iPTH) was determined by immunoradiometric analysis (IRMA) (Immunootech, Marseille, France), calcidiol (25(OH)D$_3$) and calcitriol (1,25(OH)$_2$D$_3$) by RIA methods (Immunodiagnostic Systems, Boldon, UK).

**Statistical analyses**

Results were expressed as medians (95% CI). The box-plot representations showed medians and the 25th and 75th percentile values with min–max values for the quantification of [Ca$^{2+}$]$_i$. The statistical significance of differences was tested by an independent 2-population Student’s t-test. Normally distributed data were analysed by the non-parametric Wilcoxon’s test. Spearman’s correlations between variables were used as a measure of association. A P-value of <0.05 was considered statistically significant.
Results

Comparison of free intracellular calcium in healthy subjects and CKD patients

\[\text{Ca}^{2+}\] was estimated to reach 102 (99–103) nmol/l in the cell populations of healthy volunteers while it was substantially higher in CKD patients [124 (118–126) nmol/l, \(P < 0.001\)] (Figure 1A). The effect of Tg was examined on cytoplasmic calcium concentration in both groups of studied cells. The application of Tg (1 µmol/l) in the presence of physiological extracellular Ca\(^{2+}\) concentration induced a significant increase in \[\text{Ca}^{2+}\], in CKD patients in comparison with healthy volunteers (\(P < 0.01\)) (Figure 1B and C). These findings suggest that patients in the early stage of CKD have increased intracellular calcium reserves and/or the capacitative calcium entry through calcium release-activated calcium (CRAC) channels.

Intracellular calcium reserves and capacitative calcium entry

With a view to examine the calcium concentration of intracellular Tg-sensitive stores, the effect of Tg (1 µmol/l) in the absence of extracellular calcium, chelated with EGTA (pH = 7) in the external medium, was investigated (Figure 2A). The calcium concentration of Tg-sensitive stores was significantly increased in CKD patients when compared to healthy volunteers [131 (59–311), versus 72 (50–85) nmol/l, \(P < 0.01\)] (Figure 2B). To examine the capacitative calcium entry, 2APB (50 µmol/l) was applied during the sustained phase of Tg effect and evoked a decrease in \[\text{Ca}^{2+}\], which represented Ca\(^{2+}\) influx through CRAC channels (Figure 3A). In CKD patients, the capacitative calcium entry was increased in comparison with healthy subjects [84 (48–102) versus 55 (43–77) nmol/l, \(P < 0.05\)] (Figure 3B).

Effects of vitamin D\(_3\) supplementation

Table 2 illustrates total serum calcium, ionized calcium, iPTH, 25(OH)D\(_3\) and 1,25(OH)\(_2\)D\(_3\) levels and \[\text{Ca}^{2+}\] in CKD patients at baseline and during the vitamin D\(_3\) supplementation. Serum and ionized calcium concentrations were in normal range and did not change significantly throughout the study. Initial 25(OH)D\(_3\) levels were low, and increased significantly during the treatment, although not reaching the recommended level of 30 ng/ml.

The initial median iPTH concentration was normal, but in some patients it exceeded the K/DOQI upper recommended level of 70 pg/ml for CKD stage 3 patients. During the treatment, iPTH concentrations declined continuously. At baseline, \[\text{Ca}^{2+}\] in PBMCs were increased in CKD patients when compared to healthy volunteers [123 (115–127) versus 102 (99–103) nmol/l, \(P < 0.001\)]. During the vitamin D\(_3\) supplementation, the elevated \[\text{Ca}^{2+}\] significantly decreased already at month 4 (\(P < 0.01\)). A 12-month
In this contribution, we provide evidence that \([\text{Ca}^{2+}]_i\), calcium concentration of Tg-sensitive stores and the capacitative calcium entry are significantly increased in early stages of CKD in comparison with healthy volunteers. Vitamin D3 supplementation returned the elevated \([\text{Ca}^{2+}]_i\) in CKD patients to values comparable with that in healthy subjects but did not affect intracellular calcium reserves and the capacitative calcium entry. To our knowledge, this is the first report on intracellular calcium status, dysregulation of cholecalciferol treatment returned \([\text{Ca}^{2+}]_i\), to values comparable with those in healthy volunteers (Figures 4A and 1A). Vitamin D3 treatment did not influence Tg-induced differences in \([\text{Ca}^{2+}]_i\), (Figure 4B). Intracellular calcium reserves [131 (59–311) versus 131 (41–227) nmol/l, NS] and the capacitative calcium entry [84 (48–102) versus 64 (48–82) nmol/l, NS] did not differ at baseline and after treatment, respectively.

**Table 2.** Main laboratory variables at baseline and during the cholecalciferol treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline</th>
<th>Month 4</th>
<th>Month 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total serum calcium (mmol/l)</td>
<td>2.37 (2.31–2.42)</td>
<td>2.30 (2.29–2.38)</td>
<td>2.31 (2.12–2.43)</td>
</tr>
<tr>
<td>Ionized serum calcium (mmol/l)</td>
<td>1.28 (1.26–1.32)</td>
<td>1.30 (1.28–1.34)</td>
<td>1.26 (1.24–1.30)</td>
</tr>
<tr>
<td>([\text{Ca}^{2+}]_i) (nmol/l)</td>
<td>123 (115–127)</td>
<td>113 (107–117)**</td>
<td>105 (103–112)**</td>
</tr>
<tr>
<td>1,25(OH)2D3 (pg/ml)</td>
<td>20 (16–34)</td>
<td>24 (20–37)</td>
<td>24 (17–30)</td>
</tr>
<tr>
<td>iPTH (pg/ml)</td>
<td>66 (55–101)</td>
<td>57 (51–91)*</td>
<td>55 (50–71)**</td>
</tr>
</tbody>
</table>

Values are expressed as medians (95% CI).

*P < 0.05, **P < 0.01, ***P < 0.001 for comparison with baseline and \**P < 0.01 for comparison between treatment periods.
cellular Ca\(^{2+}\) homeostasis and the effect of vitamin D\(_3\) supplementation in PBMCs of predialysis CKD patients.

[Ca\(^{2+}\)], is controlled by mechanisms that regulate Ca\(^{2+}\) entry from the extracellular environment and Ca\(^{2+}\) release from intracellular stores, and by the activity of ATP-dependent Ca\(^{2+}\) pumps and antiporters that move Ca\(^{2+}\) back into stores or out of cells. Elevation of [Ca\(^{2+}\)], is a highly versatile signal that can regulate many cellular processes. Previous studies on the metabolic profile of various cells have shown that chronic renal failure is associated with a significant elevation in [Ca\(^{2+}\)], which may be responsible for the multiple organ dysfunction in haemodialysed patients [4]. This disturbance occurs mainly due to the combination of the PTH-mediated increase in calcium entry into and the decrease in calcium exit out of the cells. Prevention of secondary hyperparathyroidism results in normalization of [Ca\(^{2+}\)], and restoration of cell function [3]. Intracellular calcium homeostasis in early stages of CKD has not been studied extensively so far. We demonstrated that [Ca\(^{2+}\)] was significantly increased already in CKD stages 2–3, while PTH levels were normal in the majority of patients.

Most of the intracellular calcium is either sequestered in the endoplasmic reticulum and mitochondria or bound to cytoplasmic proteins and other ligands. More and more evidence suggests that intracellular Ca\(^{2+}\) stores might be critical for some physiological processes [21]. Therefore, the effect of Tg, a specific inhibitor of endoplasmic reticulum Ca\(^{2+}\)-ATPase, was studied. Its application to cells results in a passive depletion of intracellular Ca\(^{2+}\) stores and thereby an activation of store-operated channels. The Ca\(^{2+}\) entering through these channels can then be pumped into the stores and thus replenish them [22]. The application of Tg to PBMCs in the presence of extracellular calcium suggests that patients in early stages of CKD have increased intracellular calcium reserves and/or calcium entry through CRAC channels. The effect of Tg in the absence of extracellular calcium strongly indicates that intracellular Tg-sensitive calcium reserves in PBMCs of CKD patients were significantly higher in comparison with healthy volunteers.

The central idea of the capacitative regulation of [Ca\(^{2+}\)], is that refilling cytosolic Ca\(^{2+}\) reserves terminates Ca\(^{2+}\) entry. To date, there are only few documented diseases that can be specifically attributed to a failure or malfunction of the capacitative calcium entry [23]. To study potential disturbances associated with the capacitative calcium entry in CKD, CRAC channels were activated by intracellular Ca\(^{2+}\) store depletion using Tg, a specific inhibitor of endoplasmic reticulum Ca\(^{2+}\)-ATPase. The increase of [Ca\(^{2+}\)], is involved in further calcium entry through calcium/cationic channels such as L-type and P2X\(_7\) channels. Therefore, 2APB, an inhibitor of CRAC channels, was applied [20]. 2APB caused a decrease in [Ca\(^{2+}\)], in PBMCs stimulated by Tg. This decrease present in cells of CKD patients was significantly higher in comparison with healthy volunteers. This finding points to increased calcium entry through CRAC channels in early CKD.

For the first time, it was also demonstrated that vitamin D\(_3\) supplementation in predialysis CKD patients is able to significantly decrease elevated concentrations of [Ca\(^{2+}\)], in PBMCs. Interestingly, these effects seemed to be independent of changes in serum levels of calciotropic hor-mones throughout the study. The role of different vitamin D metabolites in regulation of intracellular calcium homeostasis in CKD has not been elucidated. It is known that the rapid non-genomic effects of calcitriol are mediated by its binding to the vitamin D receptor (VDR) in the cell membrane and accompanied by both the calcium influx through calcium and/or cationic channels and a mobilization of calcium intracellular stores [24]. Several experimental studies have shown that at least some of these non-genomic effects in various cells may be mediated by other vitamin D metabolites including 25(OH)D\(_3\) and 24,25(OH)\(_2\)D\(_3\) [25]. Our data disclosed that the cholecalciferol treatment did not affect intracellular calcium reserves and calcium influx through CRAC channels. Recently, purinergic P2X\(_7\) receptors became the centre of interest and their possible role is now elucidated in diseases such as inflammation, hypertension, osteoporosis and kidney diseases [26–28]. Stimulation of rapid
nongenomic responses by 1,25(OH)2\text{D}3, an active metabolite of vitamin D3, induces two-step calcium response through calcium release from internal stores, followed by store refilling via calcium release-activated calcium channels (CRAC), but not L-type calcium channels in healthy human PBMCs. Furthermore, calcitriol prevents the Ca2+ increase through P2\text{X}7 channels and reduces the permeability of large molecules through the pore-forming P2\text{X}7 receptor [29]. This fact points to the potential role of P2\text{X}7 receptors in the [Ca2+]i decrease after vitamin D3 treatment. Nevertheless, further research is needed to determine whether purinergic receptors are involved in this action of vitamin D3. Whatever the mechanism of the sustained increase in [Ca2+]i in CKD may be, its normalization by cholecalciferol treatment seems promising as it could result in an improvement of function of not only PBMCs, but many other cells.

In conclusion, we demonstrated that [Ca2+]i was significantly increased in early stages of CKD. The calcium concentration of Tg-sensitive stores and the capacitative calcium entry of CKD patients were significantly higher in comparison with healthy volunteers. Vitamin D3 supplementation decreased elevated [Ca2+]i in CKD patients, but did not affect intracellular calcium reserves and calcium entry through CRAC channels under our study conditions. Further studies are necessary to elucidate the exact mechanisms of cellular calcium homeostasis regulation in CKD patients, and a relative role of vitamin D metabolites in these processes as well.

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Conflict of interest statement. None declared.

References
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