Intracellular Calcium and Sodium-Lithium Countertransport in Type 2 Diabetic Patients with and without Albuminuria

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Abstract. Increased intracellular calcium concentrations ([Ca\(^{2+}\)]) and enhanced sodium-lithium countertransport (Na/Li CT) activities may play a role in the development of diabetic complications such as diabetic nephropathy. The present study was designed to test the hypothesis that albuminuria in patients with type 2 diabetes is associated with increased [Ca\(^{2+}\)], in response to stimulation with platelet-activating factor (PAF) or with enhanced Na/Li CT activities. The study population comprised 203 type 2 diabetic patients. Albuminuria was defined as an albumin excretion rate exceeding 30 mg/d (117 cases). PAF-evoked rises in [Ca\(^{2+}\)] and Na/Li CT activities were determined in Epstein-Barr-virus-immortalized lymphoblasts. Albuminuria was related to high stimulated [Ca\(^{2+}\)] but not to high basal [Ca\(^{2+}\)]. The association was independent of age, sex and several non-diabetes related confounders, but depended on diabetes-related factors, such as the duration of diabetes. The risk of albuminuria was highest in subjects with high [Ca\(^{2+}\)], who reported a diabetes duration of ≤10 years. There was no association between Na/Li CT activities and albuminuria. The present results support the hypothesis that albuminuria in type 2 diabetic patients is associated with a primary defect in intracellular calcium homeostasis. The association between stimulated [Ca\(^{2+}\)], and albuminuria is most prominent in early diabetes.

Key words: Sodium-lithium countertransport, Intracellular calcium transients, Albuminuria, Diabetic nephropathy


DIABETES mellitus is associated with complications affecting many organ system including vasculopathy, neuropathy, retinopathy and nephropathy. Yet not all diabetic patients suffer from all of these complications or to the same pattern and degree. What causes this marked variability of susceptibility to diabetic complications is currently unknown.

It has been proposed that altered intracellular calcium metabolism plays an essential role in the pathophysiology of diabetes and its complications [1–3], but the available evidence is ambiguous. Thus, some studies reported increased basal intracellular calcium concentrations ([Ca\(^{2+}\)]) in various cell types including erythrocytes [4], platelets [5–7], B lymphocytes [8] and polymorphonuclear leucocytes [9] from patients with type 2 diabetes mellitus compared to non-diabetic controls, whereas other studies found normal basal [Ca\(^{2+}\)] in platelets [10] and skin fibroblasts [11–13] from type 2 diabetics. Several growth factors and hormones such as thrombin, bradykinin, angiotensin II, fibroblast growth factor and platelet-activating factor (PAF) are known to increase [Ca\(^{2+}\)]. Stimulated [Ca\(^{2+}\)], were normal [7] or increased [5] in platelets from type 2 diabetic patients. In some studies the effects of a stimulating factor on [Ca\(^{2+}\)] depended on the presence or absence of additional complications or risk factors. Thus, bradykinin, angiotensin II or fibroblast growth factor stimulated [Ca\(^{2+}\)] more in skin fibroblasts from type 2
diabetics with hypertension than in cells from normo-
tensive diabetics or non-diabetic controls [11, 12]. In
another study [10] thrombin-induced $[\text{Ca}^{2+}]_i$ were
higher in platelets from type 2 diabetic patients with
elevated platelet aggregation rates than in diabetics
with normal platelet aggregation.

Microalbuminuria is an early marker for diabetic
nephropathy and a major risk factor for cardiovascular
and renal disease in diabetes mellitus [14, 15]. A
sustained increase in $[\text{Ca}^{2+}]_i$ can activate a number of
potentially harmful cellular processes, including al-
tered responsiveness to hormones, dysregulated cell
proliferation and cell injury, which may contribute to
the development of albuminuria and diabetic nephrop-
athy [1, 2]. The relation between albuminuria and in-
creased $[\text{Ca}^{2+}]_i$ in type 2 diabetes has been investigated
by two studies [12, 13] using skin fibroblasts. In one
study [12] basal and fibroblast growth factor-stimulated
$[\text{Ca}^{2+}]_i$ did not depend on the presence or absence of
microalbuminuria, whereas in the other study [13] an-
giotensin II-stimulated $[\text{Ca}^{2+}]_i$ was higher in patients
with microalbuminuria than in normalalbuminuric con-
trol patients.

Diabetic nephropathy has also been reported to be
associated with an enhanced sodium-lithium counter-
transport (Na/Li CT) activity across the red cell mem-
brane in both type 1 [16–24] and type 2 diabetes [4,
25, 26]. On the other hand, several studies failed to
demonstrate enhanced Na/Li CT activities in type 1
[27–32] and type 2 diabetic patients [33–38] with
nephropathy. The issue of a potential involvement of
an increased Na/Li CT activity in the development of
diabetic nephropathy is particularly controversial in
type 2 diabetes where there are only few studies with
generally less than 100 patients involved [39].

The present study was designed to evaluate basal
$[\text{Ca}^{2+}]_i$, and the effects of direct acute stimulation with
PAF on $[\text{Ca}^{2+}]_i$ in type 2 diabetic patients with and
without albuminuria. In addition we hypothesized
that an enhanced Na/Li CT activity is related to albu-
minuria. The study was performed in immortalized
lymphoblasts. These cells offer a useful in vitro mod-
el to study primary disturbances of cellular calcium ho-
meostasis in type 2 diabetes independently of the
metabolic and hormonal abnormalities caused by the
disease.

Methods

Study population

The study population consisted of 203 patients with
type 2 diabetes mellitus who attended the hospital of
internal medicine of the University of Greifswald, the
diabetes clinic Karlsburg or the nearby community
hospital in Anklam. Assuming a mean $[\text{Ca}^{2+}]_i$ of
100 nmol/L in controls without albuminuria and a
common standard deviation of 50 nmol/L, the present
study would have been large enough to detect a mini-
imum difference in $[\text{Ca}^{2+}]_i$ between cases and controls
of 20 nmol/L with 80 percent statistical power and a
0.05 two-sided significance level (effect size $\delta = 0.4$).
The study was approved by the institutional ethical
committee of the University of Greifswald and con-
ducted according to the principles expressed in the
Declaration of Helsinki. Prior to inclusion in the study,
all patients gave written informed consent. Patients
with clinical, sonographical or laboratory evidence for
renal or urinary tract diseases other than diabetic ne-
phropathy and patients with plasma creatinine concen-
trations >150 µmol/L were excluded from the study.
Information on baseline clinical and laboratory data
were obtained from patients records. Serum hemoglobin
(Hb) A1c was determined per high-performance liquid
chromatography (Bio-Rad Diamat, Munich, Germany).
A timed urine sample was collected from each patient
and albumin excretion rate (AER) was measured using
an enzyme immunoassay (Microalbumin-ELISA EIA-
2361; DRG Instruments GmbH, Freiburg, Germany).
Albuminuria was defined as an AER >30 mg/24 h [40]
and an AER of >200 mg/24 h was considered macro-
albuminuria [41].

PAF-evoked intracellular calcium transients

Basal intracellular $[\text{Ca}^{2+}]_i$, and PAF-induced $[\text{Ca}^{2+}]_i$
were determined in immortalized lymphoblasts. From
10 ml of EDTA-treated blood lymphocytes were isolat-
ed on a Ficoll-density gradient (Lymphocyte Separa-
tion Medium, density 1.077 g/ml, Roche Diagnostics,
Mannheim, Germany). Cells were immortalized by in-
cubation with Epstein-Barr virus-containing superna-
tant from the B 95-8 cell line (Deutsche Sammlung von
Mikroorganismen und Zellkulturen GmbH, Braunsch-
weig, Germany) [42]. Cells were routinely maintained
in culture with RPMI 1640 medium (Gibco, Eggenstein,
supplemented with 2 mmol/L L-glutamine, 100 U/ml streptomycin, 100 U/ml penicillin and 10% fetal bovine serum (FBS, Gibco, Eggenstein, Germany). Passages were performed twice a week. After immortalization, lymphoblasts were grown for 16 weeks and stock cultures were frozen. For measurements of basal and stimulated \([\text{Ca}^{2+}]_i\) cells were seeded and cultured for 72 h in RPMI 1640 medium containing 10% FBS. At a final concentration of \(1 \times 10^6\) cells/ml basal and PAF-stimulated \([\text{Ca}^{2+}]_i\) were measured in Fura-2-loaded cells (Fura-2 AM from Molecular Probes, Eugene, Oregon, USA) in the presence of 1 mmol/L external \(\text{Ca}^{2+}\) in a spectrofluorometer (LS 50 B, Perkin Elmer, Überlingen, Germany) as previously described [43]. Cells were stimulated with 0.1 µmol/L PAF (Sigma, Deisenhofen, Germany). Patients were considered as having high basal or stimulated \([\text{Ca}^{2+}]_i\) when the respective values were above the top quartile of each variable.

Measurement of Na/Li CT activity

Na/Li CT activity was measured in freshly obtained erythrocytes from 520 patients. EDTA-treated blood was centrifuged at 1500 g. Red cells were washed four times with isoosmotic washing buffer [75 mmol/l MgCl₂, 80 mmol/l sucrose, 5 mmol/l glucose, 10 mmol/l tris(hydroxymethyl)aminomethane-morpholino-propane sulphoric acid (Tris-MOPS), pH 7.55] at room temperature. Red cell suspension was kept at room temperature and used within one hour. Four milliliters of the washed red cells were incubated for 15 min at 37°C with shaking in 20 ml lithium bicarbonate medium (150 mmol/l LiHCO₃, 10 mmol/l glucose, 10 mmol/l Tris-MOPS gassed with CO₂ until the pH value had adjusted to 7.55) [44]. To remove extracellular lithium cells were washed five times with buffer. Lithium efflux was measured by incubating 1.8 ml of cell suspension (haematokrit 0.05–0.08) in a sodium-free medium (150 mmol/l KCl, 1 mmol/l MgCl₂, 10 mmol/l glucose, 0.1 mmol/l ouabain, 10 mmol/l Tris-MOPS at pH 7.55) and in a sodium-containing medium (KCl in the efflux-medium was replaced by 150 mmol/l NaCl). After 60 minutes, incubation in either medium was stopped by cooling the tubes on ice for 2 min and subsequent centrifugation at 1500 g for 4 minutes at 4°C. One milliliter of the supernatant was mixed with 10 µl of 20% caesium chloride solution and the lithium concentrations were measured by atomic absorption spectrophotometry (Model Solaar 939, Unicam). Na/Li CT activity (µmol/lxh) was determined as the difference between the sodium-stimulated lithium efflux and the passive lithium efflux in the sodium-free medium over time. Enhanced Na/Li CT activity was defined as values above the top quartile of the variable.

Statistical analyses

Categorical data are presented as numbers and percentages; continuous data are presented as medians and ranges. The study population was divided into two groups according to the presence or absence of albuminuria. Differences between the two groups were compared by chi² analysis (categorical variables) or nonparametric Mann-Whitney U test (continuous variables). Logistic regression analyses were performed stepwise. First, the relation of interest was adjusted for age and sex. Second, the models were supplemented with unspecific confounders including BMI, serum LDL cholesterol and systolic blood pressure. Third, further adjustment was performed for serum HbA1c and diabetes duration as specific diabetes-related confounding variables. Fourth, a sequence of backward and forward stepwise regression analyses incorporated all two-way interaction effects between the exposure variables of interest and all other independent variables into the model. The decision to maintain an interaction was based on the likelihood ratio. Interactions were included in the models if a consistent p-value of <0.1 was achieved. Odds ratios (OR) and their 95% confidence intervals (95%-CI) are reported. For interactions, the \(\beta\) and standard error (SE) are given. Values of \(p<0.05\) were considered statistically significant. All statistical analyses were performed with SPSS software, version 11.5.1 (SPSS GmbH Software, Munich, Germany).

Results

Clinical and laboratory data of type 2 diabetic patients with and without albuminuria are outlined in Table 1. Patients in both groups were similar with respect to age, BMI, plasma creatinine as well as HDL and LDL cholesterol and triglyceride concentrations. Albuminuric patients had significantly higher HbA1c values as well as higher systolic and diastolic blood pressures than normalalbuminuric patients. The differ-
ences between the two groups with respect to gender distribution (higher proportion of women in controls) and diabetes duration (longer diabetes duration in cases) were not statistically significant. As regards medication, patients with albuminuria used more frequently insulin and calcium antagonists than patients without albuminuria. Both groups did not significantly differ with respect to therapy with sulfonylurea, biguanides, ACE inhibitors and β-blockers (Table 1).

Basal [Ca\(^{2+}\)]\(_i\) and [Ca\(^{2+}\)]\(_i\) after PAF stimulation did not differ between patients with and without albuminuria. Likewise, the differences between basal and stimulated [Ca\(^{2+}\)]\(_i\) were similar between both groups (Table 2). There were also no significant differences with respect to the number or percentage of subjects with high basal values of [Ca\(^{2+}\)]. Adjusting for age, sex and other risk factors of albuminuria did not reveal an association between high basal [Ca\(^{2+}\)]\(_i\) and albuminuria (Table 3). However, there was a higher proportion of patients with high stimulated [Ca\(^{2+}\)]\(_i\) in cases than in controls (Table 2). This relation remained stable after controlling for age, sex, BMI, serum LDL cholesterol and systolic blood pressure (Table 3). After further adjustment for diabetes duration and serum HbA1c levels.

### Table 1. Baseline characteristics of type 2 diabetic patients with and without albuminuria of >30 mg/d

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Controls n = 117</th>
<th>Albuminuria n = 86</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AER</td>
<td>mg/24 h</td>
<td>9.0 (1.3–29.4)</td>
<td>165.6 (32.0–6599.1)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Gender (female)</td>
<td></td>
<td>59.0%</td>
<td>46.5%</td>
<td>0.08</td>
</tr>
<tr>
<td>Age</td>
<td>years</td>
<td>64 (36–87)</td>
<td>65 (41–84)</td>
<td>0.83</td>
</tr>
<tr>
<td>Body mass index</td>
<td>kg/m(^2)</td>
<td>29.5 (20.0–45.1)</td>
<td>31.2 (21.0–45.9)</td>
<td>0.36</td>
</tr>
<tr>
<td>Diabetes duration</td>
<td>years</td>
<td>11.5 (0.1–40.0)</td>
<td>12.0 (0.5–49.0)</td>
<td>0.08</td>
</tr>
<tr>
<td>HbA1c</td>
<td>%</td>
<td>8.0 (4.0–12.7)</td>
<td>9.6 (5.5–12.7)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Creatinine</td>
<td>µmol/L</td>
<td>87 (39–136)</td>
<td>93 (61–141)</td>
<td>0.69</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>mmol/L</td>
<td>5.3 (2.6–13.0)</td>
<td>5.6 (1.1–8.6)</td>
<td>0.39</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>mmol/L</td>
<td>1.0 (0.6–3.0)</td>
<td>1.1 (0.6–2.8)</td>
<td>0.81</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>mmol/L</td>
<td>3.2 (0.8–6.7)</td>
<td>3.5 (1.6–5.2)</td>
<td>0.30</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>mmol/L</td>
<td>1.9 (0.6–17.0)</td>
<td>1.9 (0.2–7.0)</td>
<td>0.16</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>mmHg</td>
<td>130 (100–200)</td>
<td>140 (100–200)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>mmHg</td>
<td>76 (60–110)</td>
<td>80 (60–110)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Sulfonylurea therapy</td>
<td></td>
<td>17.5%</td>
<td>11.0%</td>
<td>0.20</td>
</tr>
<tr>
<td>Biguanide therapy</td>
<td></td>
<td>12.3%</td>
<td>18.3%</td>
<td>0.24</td>
</tr>
<tr>
<td>Insulin therapy</td>
<td></td>
<td>69.6%</td>
<td>85.4%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ACE inhibitor therapy</td>
<td></td>
<td>57.4%</td>
<td>61.0%</td>
<td>0.61</td>
</tr>
<tr>
<td>β-Blocker therapy</td>
<td></td>
<td>35.1%</td>
<td>34.1%</td>
<td>0.89</td>
</tr>
<tr>
<td>Calcium antagonist therapy</td>
<td></td>
<td>27.0%</td>
<td>46.4%</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

\*chi\(^2\)-test (categorical data) and Mann-Whitney U test (continuous data)

Data are number and percentage (categorical data) as well as median and range (continuous data).

AER denotes albumin excretion rate; HbA1c, hemoglobin A1c; BP, blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; n. s., not significantly different

### Table 2. Intracellular calcium concentrations ([Ca\(^{2+}\)]\(_i\)) of type 2 diabetic patients with and without albuminuria of >30 mg/d

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Controls n = 117</th>
<th>Albuminuria n = 86</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal [Ca(^{2+})](_i)</td>
<td>nmol/L</td>
<td>109 (55–233)</td>
<td>109 (57–347)</td>
<td>0.64</td>
</tr>
<tr>
<td>Stimulated [Ca(^{2+})](_i)</td>
<td>nmol/L</td>
<td>313 (82–3166)</td>
<td>355 (85–2189)</td>
<td>0.31</td>
</tr>
<tr>
<td>High increase of [Ca(^{2+})](_i) after stimulation</td>
<td>nmol/L</td>
<td>198 (8–3011)</td>
<td>242 (12–1975)</td>
<td>0.29</td>
</tr>
<tr>
<td>High basal [Ca(^{2+})](_i)</td>
<td>n (%)</td>
<td>27 (23.1)</td>
<td>24 (27.9)</td>
<td>0.43</td>
</tr>
<tr>
<td>High stimulated [Ca(^{2+})](_i)</td>
<td>n (%)</td>
<td>23 (19.7)</td>
<td>28 (32.6)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>High increase of [Ca(^{2+})](_i) after stimulation</td>
<td>n (%)</td>
<td>23 (19.7%)</td>
<td>28 (32.6%)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

\*chi\(^2\)-test (categorical data) and Mann-Whitney U test (continuous data)

Data are median and range (continuous data) and numbers and percentage (categorical data). High values for basal, stimulated and the difference between both [Ca\(^{2+}\)]\(_i\) were values above the top quartile of each variable.
the association between stimulated \([Ca^{2+}]\) and albuminuria was no longer statistically significant (Table 3).

In the full regression model we found one two-way interaction between high basal \([Ca^{2+}]\) and diabetes duration with respect to the risk of albuminuria (\(\beta = -1.506, SE = 0.844, p = 0.08\), Fig. 1). In the presence of high basal \([Ca^{2+}]\), the OR for the endpoint was increased by the factor 3.15 in subjects who reported a diabetes duration of \(\leq 10\) years. In persons with diabetes duration of \(>10\) years the risk of albuminuria was similar and even slightly decreased in subjects with high basal \([Ca^{2+}]\). A similar interaction as for high basal \([Ca^{2+}]\), was also found between high stimulated \([Ca^{2+}]\), and diabetes duration (\(\beta = -1.999, SE = 0.844, p = 0.02\), Fig. 2). In case of high stimulated \([Ca^{2+}]\), the OR for the endpoint rose to 5.48 in subjects with a diabetes duration of \(\leq 10\) years.

Multivariable models that also included medication as potential confounders did not substantially affect the major results. Further sensitivity analyses were performed after patients with macroalbuminuria had been excluded from the study population. Multivariable statistical models revealed similar results as given in Table 3. In the full model, there was no association between high basal \([Ca^{2+}]\), and albuminuria (OR 1.02, 95% CI 0.38; 2.73, \(p = 0.96\)), while the relation between high stimulated \([Ca^{2+}]\) bordered statistical significance (OR 2.10, 95% CI 0.86; 5.16, \(p = 0.10\)). Again, a two-way interaction was found between high stimulated \([Ca^{2+}]\), and diabetes duration (\(\beta = -1.900, SE = 0.945, p = 0.04\)).

Na/Li CT activities were similar between patients with and without albuminuria [249 (80–596) versus 222 (58–742) µmol/lxh, \(p = 0.38\)]. Both groups did also not differ with respect to the proportions of enhanced Na/Li CT activities (20.0 versus 28.1%, \(p = 0.19\)). Multivariable analyses adjusted for age, sex, BMI, systolic BP, diabetes duration, serum LDL cholesterol and serum HbA1c levels revealed no statistically significant association between enhanced Na/Li CT activity and albuminuria (OR 0.57, 95% CI 0.25; 1.33, \(p = 0.20\)). No interaction between enhanced Na/Li CT activity and the other independent variables of the full model was detected with respect to albuminuria.

**Discussion**

There is accumulating evidence that \([Ca^{2+}]\) may play an important role in the pathogenesis of diabetic complications such as vasculopathy, neuropathy, retinopathy or nephropathy [1–7, 10–13, 45]. Microalbuminuria is an early sign of incipient diabetic nephropathy and it has been postulated that derangements in intracellular calcium metabolism may contribute to this diabetic complication [1, 2]. The present findings support this hypothesis by showing that albuminuria in type 2 diabetic patients was related to high stimulated \([Ca^{2+}]\). Currently, the present study is the largest investigation dealing with this topic.

The association between albuminuria and high stimulated \([Ca^{2+}]\), was independent of a number of potentially confounding risk factors including age, sex, BMI, serum LDL cholesterol and systolic blood pressure. It was, however, confounded by diabetes-specific factors such as serum HbA1c values and diabetes duration. Thus, albuminuria was associated with high stimulated \([Ca^{2+}]\), in patients with a short (\(\leq 10\) years) but not with a long history of diabetes. This finding may indicate that in long-standing type 2 diabetes pathogenic mechanisms other than \([Ca^{2+}]\), may become increasingly important for the development of albuminuria. One of these factors may be arterial hypertension. However,
in the present study neither systolic nor diastolic blood pressure nor the presence or absence of hypertension affected the statistical association between albuminuria and high stimulated \([\text{Ca}^{2+}]_i\), indicating that the results were not confounded by different blood pressures in our groups. It is important to note that although hyperglycemia or high blood pressure could have influenced the prevalence of albuminuria in our study, these factors are unlikely to have affected basal or stimulated \([\text{Ca}^{2+}]_i\), since the use of immortalized cultured cells constitutes a strong safeguard against these potential confounders, which were either well controlled (hyperglycemia) or no longer in effect (high blood pressure) in cultured cells after several passages.

The present finding of no association between albuminuria and basal \([\text{Ca}^{2+}]_i\) in immortalized lymphoblasts is in agreement with similar studies in erythrocytes [4] and skin fibroblasts [12] from type 2 diabetic patients with and without albuminuria. Together these findings suggest that increased basal \([\text{Ca}^{2+}]_i\) do not contribute to albuminuria in type 2 diabetes.

Our findings also agree with a study [13] that found increased angiotensin II-stimulated \([\text{Ca}^{2+}]_i\), in skin fibroblast from type 2 diabetic patients with albuminuria compared to normalalbuminuric control patients. In contrast, another study [12] reported similar fibroblast
growth factor-stimulated \([\text{Ca}^{2+}]\), in skin fibroblasts from type 2 diabetic albuminuric patients and normalbuminuric control patients. The reasons for this discrepancy are unclear, but may relate to different stimulating agents and different cell types used in these [12, 13] and the present studies. Furthermore, with less than ten patients per group the previous studies [12, 13] were rather small. The finding of the present and another study [13] of an association between albuminuria and high stimulated \([\text{Ca}^{2+}]\), in type 2 diabetic patients may indicate an important role for increased intracellular calcium transients in the pathogenesis of diabetic nephropathy.

Given the potential impact of \([\text{Ca}^{2+}]\), for the prediction of diabetic nephropathy it was of interest, whether \([\text{Ca}^{2+}]\), would also be associated with other micro- and macrovascular complications in patients with type 2 diabetes. Unfortunately, we did not systematically collect data on diabetic sequelae other than nephropathy. Further studies are therefore required to investigate such associations.

In the present study, Na/Li CT activity across the red cell membrane was similar in type 2 diabetic patients with and without albuminuria. This finding is in agreement with several studies [33–38] that did not find an association between nephropathy as evidenced by the presence of albuminuria and Na/Li CT activity in type 2 diabetic patients. In contrast, other studies [4, 25, 26] reported that Na/Li CT activity was significantly increased in type 2 diabetic patients with albuminuria compared to normalbuminuric patients. The reasons for this discrepancy are unclear. It should be noticed, however, that none of the previous studies [4, 25, 26, 33–38] included more than 100 patients and only two studies [26, 34] had more than 50 patients, suggesting that lack of statistical power may have been a problem.

While in general the reproducibility of AER measurements is considered high [46], intra-individual variability of proteinuria may occur. We attempted to tackle this problem by estimating AER from 24 h urines. For practical reasons, we were only able to obtain one urine specimen for AER measurements in the present study. Thus, we cannot fully exclude that misclassification bias by day-to-day variations in proteinuria might have led to an underestimation of the effects of \([\text{Ca}^{2+}]\), and Na/Li CT activities on proteinuria in our study.

Taken together, the present results support the hypothesis that albuminuria in type 2 diabetic patients is associated with a primary defect in intracellular calcium homeostasis. The association between albuminuria and high stimulated \([\text{Ca}^{2+}]\), was particularly prominent in early diabetes and vanished with increasing diabetes duration. High stimulated rather than basal \([\text{Ca}^{2+}]\), may be particularly important as a risk factor for albuminuria in early type 2 diabetes. Na/Li CT activities are not related to albuminuria.

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