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Enhanced G protein activation in IDDM patients with diabetic nephropathy

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Summary Genetic susceptibility contributes significantly to the risk of developing nephropathy in insulin-dependent diabetes mellitus (IDDM). The cellular substrate for this has remained enigmatic. We investigated whether afflicted IDDM patients display an enhanced activation of pertussis toxin (PTX)-sensitive G proteins, a phenomenon which has been demonstrated in patients with essential hypertension. We established immortalised B lymphoblast cell lines from 10 IDDM patients without nephropathy (DC) and 15 IDDM patients with nephropathy (DN). Nephropathy was defined as a persistent albumin excretion rate of more than 20 μg/min (DC 3.9 ± 5.8, DN 562.3 ± 539.0 μg/min, respectively). Subjects were matched with regard to age (DC 28.9 ± 6.5, DN 35.9 ± 9.9 years), diabetes duration (DC 19.3 ± 6.9, DN 22.7 ± 5.8 years) and HbA_{1c} values (DC 8.5 ± 1.4, DN 8.8 ± 1.6 %). Reactivity of PTX-sensitive G proteins was quantified by measuring platelet-activating factor (PAF)-induced Ca^{2+} mobilisation (fura 2 method) and by mastoparan-stimulated [^{35}S]GTPγS binding. Expression of $G\alpha_i$ proteins was quantified by Western blot analysis. PAF-evoked Ca^{2+} increases above baseline averaged 77.0 ± 52.5 nmol/l in DC and 150.7 ± 61.5 nmol/l in DN ($p = 0.005$). PAF-evoked Ca^{2+} increases correlated with stimulated [^{35}S]GTPγS binding ($r^2 = 0.42$, $p = 0.012$). From Western blot analysis an overexpression of $G\alpha_i$ proteins could be excluded in DN. A consequence of the altered metabolic milieu in diabetes is the increased release of vasoactive and proliferative agonists which promote glomerular hyperfiltration, hypertrophy, enhanced matrix deposition, and, finally, glomerulosclerosis. Many of these auto- and paracrine agonists bind to G protein-coupled receptors. Therefore, their cellular effects are reinforced by the enhanced G protein reactivity and increase the propensity to nephropathy in IDDM. [Diabetologia (1998) 41: 94--100]

Keywords Insulin-dependent diabetes mellitus, diabetic nephropathy, G protein activation, cellular signalling, lymphoblasts, platelet-activating factor.

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Abbreviations: DN, Patients with diabetic nephropathy; DC, patients without diabetic nephropathy; GTPγ S, guanosine 5′-[γ-thio]-triphosphate; IDDM, insulin-dependent diabetes mellitus; MAS-7, Mastoparan 7; PAF, platelet-activating factor; PDGF, platelet-derived growth factor; PTX, pertussis toxin; FCS, fetal calf serum; TBS, tris-buffered saline; BSA, bovine serum albumin.

Diabetic nephropathy is now the leading cause of end-stage renal disease. About 37 % of the incidence of end-stage renal failure is attributable to diabetes mellitus [1]. Approximately 40 % of patients with insulin-dependent diabetes mellitus (IDDM) develop diabetic nephropathy (DN) during the course of their disease [2, 3], microalbuminuria being highly predictive of subsequent overt nephropathy [4]. Besides poor glycaemic control, genetic susceptibility contributes significantly to the risk of diabetic renal disease as indicated by family studies on IDDM siblings of IDDM patients [5, 6]. Furthermore, hypertension and/or a parental history of cardiovascular disease

Table 1. Characteristics of enrolled individuals

	Diabetic patients without nephropathy	Diabetic patients with nephropathy
n (male/female)	10 (4/6)	15 (6/9)
Age (years)	29 ± 7 (19--39)	36 ± 10 (27--68)
Duration of IDDM (years)	19 ± 7 (11--33)	23 ± 6 (14--34)
HbA_{1c} (%)	8.5 ± 1.4 (6.9--10.3)	8.8 ± 1.6 (6.0--11.7)
Serum creatinine (μmol/l)	84 ± 12 (71--109)	134 ± 85 (80--398)[a]
Creatinine-clearance (ml/min × 1.73 m^2)	128 ± 15 (110--158)	108 ± 48 (24--171)[a]
Albumin excretion (μg/min)	4 ± 6 (0--15)	562 ± 539 (24--1399)[a, b]
Systolic blood pressure (mm Hg)	128 ± 8 (116--140)	133 ± 22 (100--164)
Diastolic blood pressure (mm Hg)	80 ± 3 (74--84)	79 ± 13 (59--108)
Antihypertensive therapy (*n*)	0	8
Body mass index	23.1 ± 2.3 (18.4--26.4)	22.8 ± 2.9 (18.4--30.3)

Means ± SD, ranges are given in parentheses;
[a] exclusive of two patients on haemodialysis; [b] $p < 0.01$ vs patients without nephropathy

are established risk factors for DN [7, 8]. At the cellular level, an enhanced Na^+/Li^+ countertransport in erythrocytes constitutes a genetic marker for DN [9--11]. This ion transport apparently represents a special mode of operation of the ubiquitously expressed Na^+/H^+ exchanger [12]. Moreover, an increased activity of the Na^+/H^+ exchanger has been observed in erythrocytes and cultured skin fibroblasts from IDDM patients with DN [13, 14]. Subsequent investigations have revealed, that the "enhanced Na^+/H^+ exchanger phenotype"" is conserved in Epstein-Barr virus-immortalised lymphoblasts from patients with essential hypertension [15] as well as from IDDM patients with DN [16]. In hypertension, this enhanced Na^+/H^+ exchanger activity appears to be ultimately caused by an increased activation of pertussis toxin (PTX)-sensitive G proteins which results in an enhanced cellular reactivity, e. g. increased agonist-stimulated rises in $[Ca^{2+}]_i$ and increased inositol 1,4,5-trisphosphate formation, as well as an accelerated cell growth [17, 18]. Because increased proliferation has already been shown in immortalised lymphoblasts [19] and skin fibroblasts [14] from IDDM patients with DN, we speculated that enhanced G protein activation could be a common denominator of enhanced cellular reactivity in both hypertension and DN. We have, therefore, established immortalised B lymphoblast cell lines from IDDM patients with (DN) and without diabetic nephropathy (DC). The results suggest, that patients with DN actually display an enhanced activation of PTX-sensitive G proteins. This finding may help to establish a novel hypothesis regarding the pathogenesis of DN.

Subjects, materials and methods

Materials. RPMI 1640 cell culture medium, L-glutamine and penicillin/streptomycin solution were from Gibco-BRL (Eggenstein, Germany). Fetal calf serum (FCS) was from Vitromex (Vilshofen, Germany). Platelet-activating factor (PAF) and mastoparan-7 (MAS-7) were obtained from Calbiochem (Bad Soden, Germany). Fura 2-AM was purchased from Molecular Probes (Eugene, Ore., USA). Guanine nucleotides were from Boehringer Mannheim (Mannheim, Germany). [^{35}S]Guanosine 5′-[γ-thio]triphosphate ([^{35}S]GTPγS, 1200--1400 Ci/mmol) was purchased from New England Nuclear-DuPont (Dreieich, Germany). Pertussis toxin was from List Biological Laboratories (Campbell, Calif., USA). The anti-$G\alpha_{i,common}$ antibody was obtained from Calbiochem and the peroxidase-conjugated goat-anti-rabbit antibody from Sigma (Deisenhofen, Germany).

Patient characteristics. The study groups consisted of 10 patients with IDDM without nephropathy (DC) and 15 IDDM patients with nephropathy (DN), duration of IDDM being at least 10 years. The nephropathy status of the patients was determined by a timed collection of urine (collection period either 2 × 2 h, or 24 h) and measurement of the albumin concentration by immunonephelometry. Nephropathy was defined as a persistent urinary albumin excretion of more than 20 μg/min or 30 mg/24 h. The urinary albumin excretion rate was measured at least two times for each patient. Estimates of the endogenous creatinine clearance were performed either as a 2 × 2 h or a 24 h creatinine clearance. All subjects had repeated measurements of blood pressure after 10 min of rest and antihypertensive medication was recorded. A family history of diabetes, hypertension, myocardial infarction, other cardiovascular diseases, stroke and hyperlipoproteinaemia was taken.

Characteristics of the two study groups are summarised in Table 1. IDDM patients with and without DN did not differ significantly regarding age, duration of diabetes, gender, glycaemic control and body mass index. However, both groups differed with respect to the markers of renal disease. DN patients included six with microalbuminuria, seven with macroalbuminuria and two already on haemodialysis. Renal disease was also reflected by higher serum creatinine values in the DN group. In addition, hypertension was diagnosed more often in the DN group as reflected by antihypertensive treatment. Of 15 DN patients 8 were on antihypertensive medication, whereas none of the 10 DC patients was treated with antihypertensives. Mean systolic and diastolic blood pressures were similar in both groups, although the diastolic blood pressure values in some of the patients in the DN group exceeded normotensive values. Additionally, in the DN group a family history of cardiovascular disease was reported more often (data not presented).

Lymphoblast culture. Fifty ml of blood was obtained from each patient, lymphocytes were isolated on a Ficoll-Diatrizoate

gradient (Ficoll Paque; Pharmacia, Freiburg, Germany) and immortalised as described [15]. Cells were routinely maintained in RPMI 1640 medium supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 % FCS. Measurements of intracellular signal transduction were not started before the 12th--16th week after immortalisation. Repeated measurements were performed for agonist-stimulated increases in $[Ca^{2+}]_i$ and GTPγS binding with a minimum interval of 6 weeks between measurements to avoid fortuitous influences of the cell culture conditions.

Ca^{2+}-measurements. Measurements of intracellular free calcium concentration ($[Ca^{2+}]_i$) were performed on cells seeded at 1×10^5 cells/ml and cultured for 24 h in serum-free RPMI 1640 medium. Cells resuspended at 2×10^6 cells/ml in serum-free medium were incubated with 3 μmol/l fura 2-AM for 30 min at 37 °C. Thereafter, the cells were centrifuged and resuspended for an additional 30 min in RPMI 1640 medium containing 1 mmol/l Ca^{2+} to allow for complete hydrolysis of the fluorescent dye. Before each single measurement, aliquots of cells ($0.5\text{--}1 \times 10^6$ cells) were transferred to Eppendorf tubes, briefly centrifuged and resuspended in 2 ml pre-warmed HEPES buffer containing (in mmol/l): 135 NaCl, 5 KCl, 1 MgCl, 1 $CaCl_2$, 10 Glucose, and 20 HEPES, pH 7.4. Measurements were performed using an LS 5B spectrofluorometer (Perkin Elmer Corp., Norwalk, Conn., USA) equipped with a thermostated cuvette holder and a fast-filter application. Cells were excited alternately at 340/380 nm, and emission was recorded every 0.1 s at 495 nm. For experiments under Ca^{2+}-free conditions, 5 mmol/l EGTA (final concentration) was added to the cell suspension 30 s before addition of agonist. $[Ca^{2+}]_i$ was calculated as described [20]. On the day of the experiment, cells from each individual cell line were measured 3 to 7 times for each condition, and these measurements were repeated two to three times for each cell line several weeks or even months apart.

[^{35}S]GTPγS binding. Determination of [^{35}S]GTPγS binding to permeabilised cells was performed as described [21]. Lymphoblasts were growth-arrested as described above. After centrifugation ($120 \times g$), the cell pellet was resuspended at 1×10^7 cells/ml in a buffer containing (in mmol/l) 150 NaCl, 5 $MgCl_2$, 1 EDTA, and 50 triethanolamine-HCL, pH 7.4. Aliquots (1×10^6 cells) were transferred to the reaction mixture consisting of the same buffer supplemented with 10 μmol/l digitonin, 100 μmol/l adenosine 5′-[β, γ-imino] triphosphate and 10 μmol/l guanosine diphosphate (GDP), and the cells were permeabilised for 15 min at 30 °C. Thereafter, 30 μmol/l mastoparan-7 (MAS-7) was added. After 1 min, the [^{35}S]GTPγS binding reaction was started by addition of 10 nmol/l [^{35}S]GTPγS (0.1 μCi/tube). The reaction was stopped after 10 min by rapid filtration through nitrocellulose filters rinsed four times with 3 ml of ice-cold washing buffer consisting of 5 mmol/l $MgCl_2$ and 50 mmol/l Tris-HCL (pH 7.5) for separation of protein-bound and free radioactivity. The filters were counted in a liquid scintillation spectrometer. Non-specific binding was defined as that not competed for by 10 μmol/l unlabelled GTPγS. Experiments were performed on cell lines from five DC and nine DN patients because the method was too difficult to be performed on all cell lines simultaneously. This would have been necessary to obviate interassay variations.

Immunoblotting. Crude lymphoblast cell membranes were prepared by nitrogen cavitation as described [17]. Cells seeded at a density of 1×10^6 cells/ml in serum-containing RPMI 1640 medium and grown for 1 day were harvested, washed twice in phosphate-buffered saline (PBS), resuspended in ice-cold lysis buffer (in mmol/l: 250 sucrose, 1.5 $MgCl_2$, 1 ATP, 3 benzamidine, 1 phenylmethylsulfonyl fluoride, 2 μg/ml soybean trypsin inhibitor, and 20 Tris/HCl, pH 7.5), and homogenised by nitrogen cavitation (15 min at 50 bar). The cavitate was centrifuged at $2500 \times g$ for 10 min, and a crude membrane fraction was obtained from the resulting supernatant by centrifugation at $100\,000 \times g$ for 20 min. The resulting pellet was washed in a buffer consisting of (in mmol/l) 1 EDTA, 1 dithiothreitol, 3 benzamidine, 1 phenylmethylsulfonyl fluoride, 2 μg/ml soybean trypsin inhibitor, and 20 Tris/HCl, pH 7.5, resuspended, and finally stored in the same buffer at --70 °C.

Protein concentration was determined according to Bradford [22] with bovine IgG as standard. Membrane proteins (25 μg) were heated for 5 min at 95 °C in sample buffer containing 5 % 2-mercaptoethanol. They were fractionated by SDS-PAGE electrophoresis according to Laemmli [23] with 10 % acrylamide in the running gel and 5 % acrylamide in the stacking gel. Proteins were electrotransferred to nitrocellulose using a Trans-Blot electrophoretic transfer cell (Biorad, München, Germany) at 100 V for 1 h. Nitrocellulose filters were blocked for 1 h at room temperature in Tris-buffered saline (TBS: 10 mmol/l Tris-HCl pH 8.0, 150 mmol/l NaCl) containing 5 % bovine serum albumin (BSA) and 0.1 % Tween 20. Thereafter, the filters were washed three times for 5 min each in TBS containing 0.05 % Tween 20 (TBS/Tween 20) and subsequently incubated for 60 min at room temperature with the primary antibody (anti $G\alpha_{i,common}$) which was diluted 1:1000 with TBS containing 0.1 % BSA (TBS/BSA). The filters were washed four times for 5 min each in TBS/Tween 20 and incubated for another hour at room temperature in the peroxidase-conjugated antibody which was diluted at 1:5000 with TBS/BSA. The filters were treated with enhanced chemoluminescent (ECL)-reagent (Amersham, Braunschweig, Germany) according to the manufacturer and immunoreactive bands were visualised by autoradiography.

Statistical analysis. All data are given as mean ± SD if not indicated otherwise. Comparison between groups were made using Student's *t*-test or, if appropriate Mann-Whitney U test. Differences were regarded as significant at p less than 0.05. All calculations were performed using the computer program StatView, version 4.0 for Macintosh, Abacus Concepts, Inc. (Berkeley, Calif., USA).

Results

PAF-stimulated increases in $[Ca^{2+}]_i$. Mean values of basal $[Ca^{2+}]_i$ in the presence of 1 mmol/l external Ca^{2+} were not significantly different in lymphoblasts from both groups (DC: 84 ± 27 nmol/l, $n = 83$ measurements; DN: 87 ± 27 nmol/l, $n = 165$ measurements). Addition of 0.1 μmol/l PAF consistently induced a rapid, transient increase in $[Ca^{2+}]_i$, which peaked within 15 to 30 s and then declined to a new steady state level above baseline (Fig. 1A, B). We observed differences in the increase in $[Ca^{2+}]_i$ above basal levels between lymphoblasts from DN, and lymphoblasts from DC patients, which also resulted in different sizes of the areas under the curve (Fig. 1A, B). Figure 2A depicts the mean increases in $[Ca^{2+}]_i$ above basal values for different cell lines upon stimulation with 0.1 μmol/l PAF in the presence

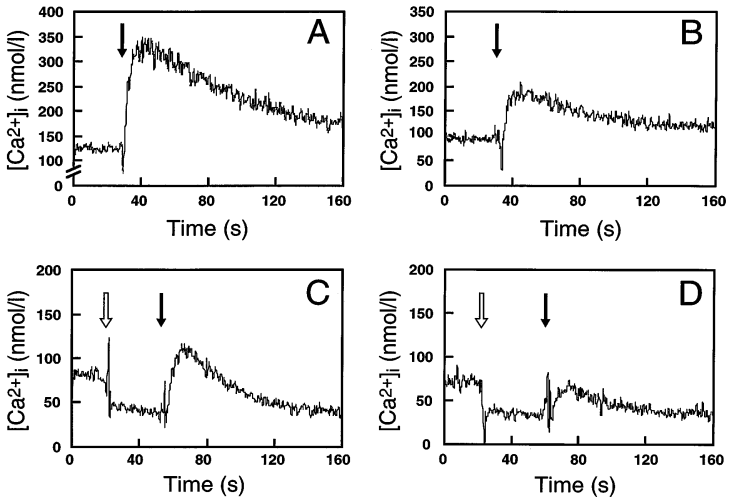

Fig. 1. Representative tracings of changes in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) induced by platelet-activating factor (PAF) in human lymphoblast cell lines from two IDDM patients, one having developed diabetic nepropathy (**A**, **C**), the other without (**B**, **D**). Serum-deprived lymphoblasts loaded with fura 2-AM and incubated in HEPES buffer were exposed to 0.1 μmol/l PAF (solid arrows) in the presence of 1 mmol/l extracellular Ca^{2+} (**A**, **B**), and after chelation of extracellular Ca^{2+} ions with 5 mmol/l EGTA (transparent arrows; **C**, **D**)

of 1 mmol/l external Ca^{2+}. In the DC group, 0.1 μmol/l PAF induced a mean increase in $[Ca^{2+}]_i$ by 77.0 ± 52.5 nmol/l above baseline (n = 10), whereas in the DN group the mean increase above baseline amounted to 150.7 ± 61.5 nmol/l (n = 15). This difference was statistically significant (p = 0.005). A difference in the slope of the rise or decline in Ca^{2+} mobilisation between the two groups was not noted (Fig. 1A, B).

Upon chelation of extracellular Ca^{2+} ions by addition of 5 mmol/l EGTA to the cell suspension, basal $[Ca^{2+}]_i$ values fell to 39 ± 17 nmol/l (n = 72) in DC and 41 ± 15 nmol/l (n = 126) in DN cell lines. The subsequent stimulation of the cells with 0.1 μmol/l PAF still induced an increase in $[Ca^{2+}]_i$ (Fig. 1C, D), which ranged from 0--100 nmol/l in DC cell lines and from 30--140 nmol/l in DN cell lines (Fig. 2B). The mean increase in $[Ca^{2+}]_i$ of 38.0 ± 25.7 nmol/l in the DC group and 70.0 ± 29.0 nmol/l in DN was significantly different (p = 0.0097).

Subdivision of the DN group into patients with (DN-HT) or without (DN-NT) antihypertensive treatment revealed differences in the increase in $[Ca^{2+}]_i$, the values for the subgroups being 114.3 ± 45.0 nmol/l for DN-NT (n = 7) and 182.5 ± 57:8 nmol/l for DN-HT (n = 8) in the presence of extracellular Ca^{2+} (Fig. 2A); the Δ $[Ca^{2+}]_i$ values in the absence of extracellular Ca^{2+} being 58.6 ± 22.7 nmol/l and 80.0 ± 31.6 nmol/l, respectively (Fig. 2B). These differences reached statistical significance only in the presence of extracellular Ca^{2+} (p = 0.026 and p = 0.16, respectively).

Correlation of MAS-7-stimulated [^{35}S]GTPγS binding and PAF-evoked $[Ca^{2+}]_i$ rises. To investigate whether the magnitude of the PAF-induced increases in $[Ca^{2+}]_i$ correlates with the activation of PTX-sensitive G_i proteins, we determined the MAS-7-stimulated binding of [^{35}S]GTPγS to permeabilised lymphoblasts from DC and DN. The peptide MAS-7 mimics the configuration of an activated G protein-coupled receptor and is, therefore, used as a direct activator of G proteins in the absence of receptor agonists [24]. The addition of MAS-7 (30 μmol/l) to permeabilised lymphoblasts stimulated [^{35}S]GTPγS binding by 60--276 % above control in the 14 different B lymphoblast cell lines (5 DC and 9 DN) investigated. The mean stimulation above control was 122.8 ± 86.1 % for DC and 161.8 ± 70.8 % for DN. Although there was a tendency in DN to an enhanced stimulation of [^{35}S]GTPγS binding compared to DC, this difference did not reach statistical significance (p = 0.38). Nevertheless, when the PAF-induced increases in $[Ca^{2+}]_i$ were plotted against MAS-7 stimulated binding of [^{35}S]GTPγS (Fig. 2), linear regression analysis revealed a significant correlation between the direct activation of G_i type G proteins by MAS-7 and the PAF-induced increases in $[Ca^{2+}]_i$ (r^2 = 0.42; p = 0.012).

$G\alpha_i$ protein expression. To investigate whether enhanced G protein activation in B lymphoblasts from DN is caused by an overexpression of PTX-sensitive G proteins, the expression of $G\alpha_i$ was quantified by Western blot analysis. Figure 3 displays a representative analysis of $G\alpha_i$-subunit expression as assessed using an anti-$G\alpha_{i,common}$ antibody. This antibody detected a single band at about 42 kDa. Densitometric analysis demonstrated a similar density for DC (46.71 ± 8.89 arbitrary units; n = 4) and DN (50.12 ± 17.98 arbitrary units; n = 5).

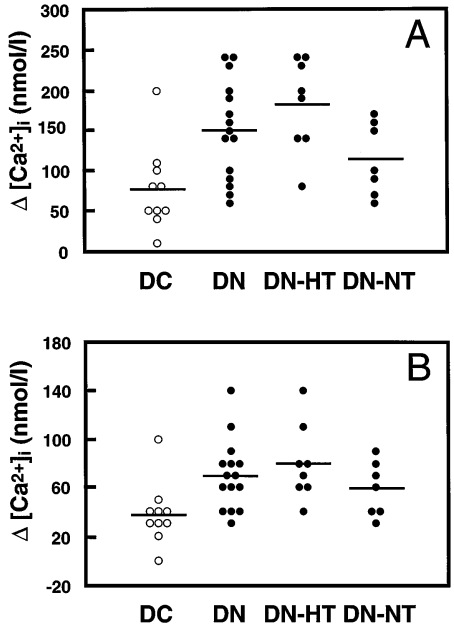

Fig. 2. Platelet-activating factor (PAF, 0.1 μmol/l)-induced rises in cytosolic free calcium concentration above baseline ($\triangle [Ca^{2+}]_i$) in lymphoblasts from IDDM patients without (DC) and with (DN) diabetic nephropathy. The DN group was subdivided into patients who were treated with antihypertensives (DN-HT) or not (DN-NT); **A** in the presence of 1 mmol/l extracellular Ca^{2+} ($p < 0.01$, DN vs DC; $p < 0.05$, DN-HT vs DN-NT); **B** in the absence of extracellular Ca^{2+} after chelation with 5 mmol/l EGTA ($p < 0.01$, DN vs DC)

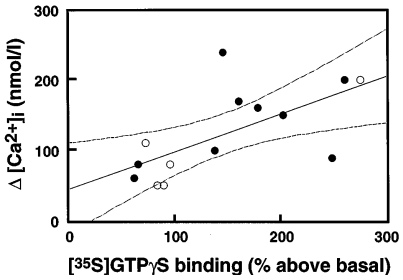

Fig. 3. Correlation between G protein activation expressed as mastoparan-7 (M-7, 30 μmol/l) stimulated binding of $[^{35}S]GTP\gamma S$ to permeabilised lymphoblasts and PAF (0.1 μmol/l)-induced increase in cytosolic free calcium concentration ($\triangle [Ca^{2+}]_i$) in lymphoblasts from diabetic patients without (○) and with (●) diabetic nephropathy ($r^2 = 0.42$; $p = 0.012$)

Discussion

We have established immortalised B lymphoblast cell lines from IDDM patients with and without DN. This experimental approach allows determination of potentially inherited abnormalities of intracellular signal transduction because confounding influences of the hyperglycaemic diabetic in vivo mileu are very unlikely to persist after prolonged cell culture. Subsequently, we quantified the PAF-induced increase in $[Ca^{2+}]_i$, which is predominantly mediated via PTX-sensitive G_i-type G proteins in B lymphoblasts [17, 25]. Lymphoblasts from IDDM patients with DN displayed significantly higher increases in $[Ca^{2+}]_i$ above baseline compared to lymphoblasts from IDDM patients without DN both in the presence and absence of extracellular Ca^{2+} ions. On the other hand, we found no correlation between the duration of IDDM or donor age and the increase in $[Ca^{2+}]_i$ (data not shown and [26]). Agonist-evoked increases in $[Ca^{2+}]_i$ correlate reasonably well with the activation of G_i proteins as inferred from MAS-7 stimulated binding of $[^{35}S]GTP\gamma S$ to permeabilised lymphoblasts. The fact that differences in stimulated $GTP\gamma S$ binding between DN and DC cell lines did not reach statistical significance is mainly due to the restricted number of respective determinations that could be performed under comparable experimental conditions. The enhanced activation of G_i-type G proteins in DN is not caused by an overexpression which resembles our previous observation in cell lines from hypertensive subjects [17, 18]. Hence, the molecular reason for this remains to be unravelled.

It should be emphasised that the present study was not intended to be an epidemiological one. Immortalisation and prolonged culture of cell lines from large numbers of IDDM patients is desirable but hardly feasible. A striking similarity exists between the findings reported here and those published previously on immortalised lymphoblasts from IDDM patients with DN and patients with essential hypertension (without IDDM), which in our view, makes the present observations highly plausible: Thus, the phenotype of enhanced Na^+/H^+ exchanger activity persists in immortalised B lymphoblasts [16, 19] and skin fibroblasts [13, 14] from DN patients and patients with hypertension [15] and is tightly linked to an accelerated cell proliferation. Both B lymphoblasts as well as skin fibroblasts from hypertensive patients with enhanced Na^+/H^+ exchanger activity display a selective enhancement of activation of G_i-type G proteins [17, 18]. It is, therefore, not too surprising that the same phenomenon, i.e. increased G_i protein activation, is now also described in IDDM patients with DN. Furthermore, the findings presented here are supported by in vivo studies: IDDM patients with DN respond with an increased vasoconstriction to infusion of clonidine, which activates α_2-adrenoceptors coupled to G_i proteins, whereas vasoconstriction in response to the α_1-adrenoceptor agonist phenylephrine, which activates PTX-insensitive G proteins, is not significantly different in IDDM patients with and without DN [27]. However, the striking similarities in terms of increased cellular signalling in both hypertension and DN may also cause some concern regarding a potential selection bias in this study and those performed by others [16, 19]. This concern relates to the relative excess of patients with hypertension in the DN group compared to the low number

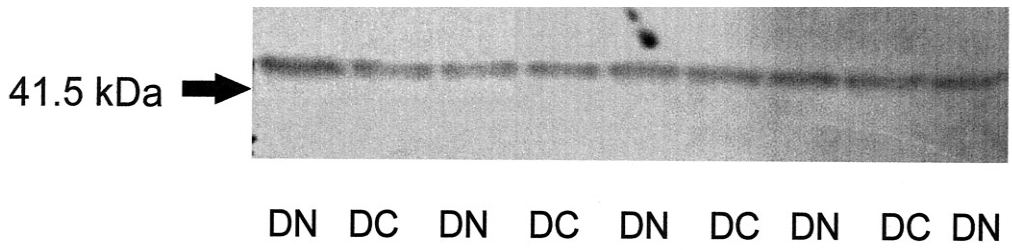

Fig. 4. Representative Western blot analysis of G protein subunit expression in lymphoblast membranes from 4 DC and 5 DN patients. Blots were probed with an antibody directed against $G\alpha_{i,common}$

of hypertensive individuals in the DC group both in the present as well as in previous studies [16, 19]. It is, therefore, at present difficult to decide, whether abnormal cellular signal transduction is a typical feature and a potential pathogenetic mechanism for DN or just a reflection of the large number of hypertensive individuals who unavoidably accumulate among IDDM patients with DN. On the other hand, an enhanced activation of G_i proteins could represent the cellular substrate of a common genetic predisposition for both hypertension and, in the hyperglycaemic condition of IDDM, the development of DN.

Based on our findings, we should like to propose an alternative hypothesis regarding the pathogenesis of nephropathy in IDDM. High extracellular glucose concentration induces an increased de novo synthesis of diacylglycerol (DAG) in the kidney [28] which in turn activates protein kinase C and phospholipase A_2 [29, 30]. This results in the synthesis and release of multiple autocrine and paracrine growth factors as well as lipid mediators involved in the pathogenesis of diabetic nephropathy from early changes to the late stage of irreversible kidney damage.

Glomerular hyperfiltration is the first functional alteration during the early stage of the disease. Alterations in mesangial contractility, which can be caused by the action of PAF and lysophosphatidic acid [31, 32] and an enhanced vasoconstrictor effect of angiotensin II on the vas efferens have been proposed among others [33]. These actions of PAF, lysophosphatidic acid and angiotensin II [34] are largely mediated by PTX-sensitive G proteins in the kidney. Angiotensin II not only stimulates phospholipase C and A_2, which results in the release of arachidonic acid and an increase in prostaglandin E_2 [35], but also stimulates extracellular matrix protein synthesis and cellular hypertrophy in glomerular mesangial and proximal tubular cells by the autocrine induction of transforming growth factor β (TGF-β) and platelet-derived growth factor (PDGF) [36--38]. PDGF and TGF-β seem to be directly involved in the initiation of cellular hypertrophy, matrix and collagen expression, and glomerulosclerosis. The cellular actions of PDGF are partially mediated by PTX-sensitive G proteins [39, observations from our laboratory].

The above-described mechanisms induced through elevated glucose concentrations are already detrimental in IDDM patients with "normal"" activation of PTX-sensitive G proteins. Patients with enhanced activation of PTX-sensitive G proteins, however, would experience a much more vigorous cellular activation by the above-mentioned compounds. Thus, typical diabetic complications such as DN may develop much earlier and/or be more pronounced during the natural course of IDDM.

In summary, our data apparently support the hypothesis of an inherited predisposition to DN in IDDM patients. This may be due to an increased activation of PTX-sensitive G proteins, which are key mediators in pathogenic processes of this major complication of IDDM. So far, the enhanced activation of G_i proteins may serve as an intermediate phenotype for IDDM patients at risk for developing diabetic nephropathy, until a true genetic marker has been identified.

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