Effect of protein kinase C modulators on the leucocyte Na^+/H^+ antiport in Type 1 (insulin-dependent) diabetic subjects with albuminuria

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Summary. It is uncertain why only one third of Type 1 (insulin-dependent) diabetic patients develop nephropathy. One suggestion is the inheritance of a predisposition to essential hypertension. We have previously found elevated Na^+/H^+ antiport activity and a raised intracellular pH in leucocytes from hypertensive and Type 1 diabetic subjects with albuminuria using a novel double ionophore fluorimetric technique. These changes are not found in Type 1 diabetic subjects without albuminuria. We wished to test the effect of a protein kinase C inhibitor staurosporine (100 nmol/l) on the elevated antiport activity, and the degree of stimulation achieved by exogenous diacyl glycerol. Raised leucocyte Na^+/H^+ antiport activity of Type 1 diabetic subjects with albuminuria ($73.8 \pm 17.2 \text{ mmol} \cdot \text{l}^{-1} \cdot \text{min}^{-1}$) was restored to normal levels with staurosporine ($54.9 \pm 17.9 \text{ mmol} \cdot \text{l}^{-1} \cdot \text{min}^{-1}$, $p < 0.001$). The leucocyte Na^+/H^+ antiport activity of diabetic subjects without albuminuria fell significantly also

with staurosporine but to a lesser extent (57.3 ± 11.6 to $50.0 \pm 12.8 \text{ mmol/l}$, $p < 0.003$). In contrast, leucocytes from normal control subjects showed no change in antiport activity with staurosporine (54.3 ± 8.5 to $52.6 \pm 10.4 \text{ mmol} \cdot \text{l}^{-1} \cdot \text{min}^{-1}$). Dioctanoyl glycerol stimulated the leucocyte Na^+/H^+ antiport in normal subjects and diabetic patients without albuminuria, with significantly less stimulation in diabetic patients with albuminuria. We conclude that reversal by staurosporine of the elevated Na^+/H^+ antiport activity in Type 1 diabetic subjects with albuminuria could indicate a role for protein kinase C in activating the antiport. This hypothesis is supported by the reduced stimulation of the antiport by dioctanoyl glycerol in this group of patients.

Key words: Sodium, leucocytes, amiloride, protein kinase, Type 1 (insulin-dependent) diabetes.

It is not at present known why nephropathy develops in only about one third of Type 1 (insulin-dependent) diabetic subjects [1]. Poor metabolic control may play an important role in the development of albuminuria [1–3], but the development and progression of nephropathy may not be dependent only on the level of glycaemic control [2, 3]. The involvement of genetic factors is suggested by the higher frequency of albuminuria in diabetic siblings of diabetic patients with rather than without albuminuria [4]. Furthermore, the risk of developing nephropathy in Type 1 diabetes is increased with a parental history of essential hypertension [5]. Also, the blood pressure of non-diabetic parents of Type 1 diabetic patients with nephropathy is higher than that of non-diabetic parents of diabetic subjects without nephropathy [6]. Predisposition to hypertension may be an additional factor that contributes to the development of nephropathy.

Many membrane transport defects have been described in hypertension [7], but the most reproducible defect is an elevated erythrocyte Li^+/Na^+ exchange [8]. This membrane transport defect is strongly determined by genetic factors [9] and may represent susceptibility to hypertension. Two groups [5, 10] subsequently demonstrated elevated erythrocyte Li^+/Na^+ countertransport in Type 1 diabetic patients with nephropathy, suggesting that inheritance of a trait conferring susceptibility to hypertension may also increase the risk of developing diabetic renal disease. Since Li^+ is not found intracellularly at the levels used in these transport assays, it has been suggested that Li^+/Na^+ exchange may be mediated via the Na^+/H^+ antiport [11].

We and others have previously reported an elevated Na^+/H^+ antiport activity in human essential hypertension [12–15] and the spontaneously hypertensive rat [16,

17]. One possible reason for this elevated antiport activity could be increased protein kinase C activity [15], itself perhaps secondary to elevated phospholipase C activity [18]. Phospholipase C acts on membrane phosphoinositides, releasing inositol triphosphate, which raises intracellular free calcium, and membrane bound diacyl glycerol, which activates protein kinase C. We subsequently reported that leucocyte Na⁺/H⁺ antiport activity and intracellular pH were both elevated in Type 1 diabetic subjects with albuminuria, but were in the normal range in those without albuminuria [19]. In this present study, we wished to test the hypothesis that this elevated leucocyte Na⁺/H⁺ antiport activity was secondary to protein kinase C activation, similar to the changes found in essential hypertension. The effects of staurosporine (an inhibitor of protein kinase C [20] and other protein kinases) and dioctanoyl glycerol (a synthetic diacyl glycerol which activates protein kinase C) on Na⁺/H⁺ antiport activity were determined in Type 1 diabetic subjects with and without albuminuria and in matched normal control subjects.

Subjects, materials and methods

Materials

Nigericin, monensin, fatty-acid free bovine serum albumin, dioctanoyl glycerol and tissue culture fluid (TC199) were from the Sigma Chemical Co., (Poole, Dorset, UK). TC199 was buffered with 15 mmol/l 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) and was nominally HCO₃⁻ free. Ionic composition was adjusted to Na⁺ 140 mmol/l, K⁺ 5.5 mmol/l (pH 7.4 with Tris base). Ethyl-isopropyl amiloride (EIPA) was a gift from Dr. Alexander Bearn, Merck, Sharpe and Dohme, (Rahway, NJ, USA). Bis(carboxyethyl)carboxyfluorescein pentaacetoxymethyl ester (BCECF-AM) and staurosporine were from Cambridge Bioscience, (Cambridge, UK).

Subjects

Fifteen Type 1 diabetic subjects without albuminuria and 19 diabetic patients with urinary albumin/creatinine ratios (mg/mmol) greater than 2 were recruited for the study. They were compared with 16 normal control subjects. These three groups were matched for age and body mass index, and the two diabetic groups were matched for duration of diabetes. All subjects were European and had normal plasma creatinine and electrolytes. None were on the oral contraceptive pill, beta blockers, calcium antagonists, diuretics of any sort or digoxin. They were on an unrestricted salt intake and none of the subjects were on weight reducing diets. Permission was granted for the study from the Central Oxford research ethics committee.

Urine and plasma measurements

Subjects collected early morning urine specimens for three consecutive days for analysis of creatinine, sodium and potassium. Urine albumin was measured by immunoturbidimetry [21]. The three urine albumin/creatinine ratios were averaged and ratios above 2.0 mg/mmol were considered elevated so that the sensitivity of detection of microalbuminuria was above 95% [22]. None of the sub-

jects had a urinary tract infection nor any systemic infection for at least two months prior to the study.

Subjects fasted overnight from 22.00 hours and omitted their morning dose of insulin on the day of the study. On arrival at the laboratory, a cannula was inserted into an antecubital vein under local anaesthetic. After 30 min bed rest, supine blood pressure was measured in triplicate from the right arm with a Hawksley random zero sphygmomanometer by one of two observers. Eighty ml blood was taken for measurement of plasma creatinine and electrolytes, glucose (by a glucose oxidase method on a Beckman glucose analyser, Fullerton, Calif., USA), glycated haemoglobin (by agar gel electrophoresis, Corning Chemical, Palo Alto, Calif., USA) and leucocyte Na⁺/H⁺ antiport studies. After immediate centrifugation 5 ml blood was subjected to polyethylene glycol to precipitate the antibody bound insulin. The supernatant was stored at -20°C for later determination of plasma free insulin using radioimmunoassay [23].

Leucocyte measurements

Intracellular pH, buffering power and Na⁺/H⁺ fluxes. Leucocytes were isolated from the blood by dextran sedimentation and residual erythrocytes lysed by hypotonic shock [24]. Cells were then resuspended in tissue culture fluid (TC199) with 10% autologous serum. One aliquot of the leucocyte pellet was treated with 100 nmol/l staurosporine in TC199 for 1 h. Another aliquot was left in TC199 at 37°C for a similar period of time. Both cell aliquots were then loaded with BCECF by incubating in TC199 with 10 µmol/l BCECF-AM [25–27]. After 30 min incubation at 37°C, the cells were spun down and washed three times before resuspending in TC199. The dye-loaded cells were left at room temperature for at least 30 min before any readings were made. Staurosporine-treated cells were kept with the inhibitor during the stage of dye loading and at all subsequent stages of the experiments. Leucocyte Na⁺/H⁺ antiport fluxes determined immediately after dye loading were similar to those measured 8 h later (unpublished observations).

Resting intracellular pH was studied by suspending untreated and staurosporine-treated cells in a buffer of the following composition (in mmol/l): NaCl 140, KCl 5, CaCl₂ 0.8, MgSO₄ 0.8, HEPES 15, glucose 5, bovine serum albumin (1 g/l), pH 7.4 at 37°C. Fluorescence emission of BCECF was measured at 530 nm with excitation at 500 and 439 nm as previously described [25–27] with 10⁶ cells/ml buffer in a Perkin Elmer luminescence spectrometer (LS-5B). The ratio 500/439 nm is a measure of intracellular pH and is independent of dye concentration, photobleaching or cell density [25, 26]. Buffering power was determined by addition of 2–4 mmol/l (final concentration) of (NH₄)₂SO₄ and measuring the change in intracellular pH [26–28]. Buffering power is defined as:

Change in [NH₄⁺]_{in} / Change in internal pH
The internal NH₄ concentration [NH₄⁺]_{in} is calculated as

$$\frac{\text{Total base concentration} \times 10^{(\text{pH}_o - \text{pH}_i)}}{1 + 10^{(\text{pH}_o - \text{pK})}}$$

where total base concentration is 2–4 mmol/l, pH_o and pH_i are the external and internal pH values after the addition of (NH₄)₂SO₄, and pK is 8.89 at 37°C. The units for buffering power are in mmol·l⁻¹·pH U⁻¹.

Intracellular pH was clamped at pH 6.0 as previously described [26, 27]. Briefly, cells were incubated for 5 min at 37°C in a buffer composed of (in mmol/l) KCl 140, CaCl₂ 1.8, MgSO₄ 0.8, glucose 5, HEPES 15, and the 2 ionophores (in µmol/l) nigericin 2, monensin 5, pH 6.0 at 37°C with Tris base. The ionophores set intracellular pH to equal external pH, and concurrently deplete intracellular Na, preventing drift of the intracellular pH subsequently [26, 27]. The ionophores were then removed by washing in a buffer identical to that above but without the ionophores and which contained 1 g/l fatty-acid free bovine serum albumin. The albumin binds the ionophores and extracts them from the cell membranes. On the addition of 1.9 ml of isotonic NaCl buffer to 0.1 ml of the cell suspen-

Table 1. Clinical characteristics and plasma measurements of the subjects in the study. Means and 1 SD are reported except for plasma free insulin and urine albumin/creatinine ratios, where geometric mean [geometric mean + 1 SD] are given

	Type 1 (insulin-dependent) diabetic subjects		Normotensive control subjects
	Albuminuria	No albuminuria	
Number (male)	19 (16)	15 (7)	16 (10)
Body mass index (kg/m ²)	23.7 ± 2.8	23.4 ± 2.3	22.3 ± 2.1
Age (years)	33 ± 11	37 ± 12	33 ± 13
Systolic blood pressure (mm Hg)	129 ± 18 ^a	127 ± 12 ^a	118 ± 9
Diastolic blood pressure (mm Hg)	78 ± 11	73 ± 6	77 ± 7
Duration of Diabetes (years)	20.6 ± 8.7	23.1 ± 10.8	–
Insulin dose (U/day)	52.2 ± 16.3	47.4 ± 14.9	–
Fasting plasma glucose (mmol/l)	15.1 ± 5.2 ^b	12.5 ± 4.2 ^b	4.9 ± 0.3
Fasting plasma free insulin (mU/l)	8.0 [16.7]	7.9 [11.7]	8.6 [14.1]
Plasma creatinine (μmol/l)	90 ± 14	79 ± 10	84 ± 13
Glycated haemoglobin (%)	9.9 ± 1.8 ^b	9.5 ± 2.2 ^b	6.0 ± 0.5
Urine albumin: creatinine ratio	12.7 [45.7] ^b	0.4 [1.2] ^c	0.2 [0.5]

^a $p < 0.05$; ^b $p < 0.001$ compared to normal control subjects^c $p < 0.001$ compared to diabetic patients with albuminuria

sion in isotonic KCl buffer (final concentrations, in mmol/l, NaCl 133, KCl 5; final extracellular pH 7.4), the intracellular H⁺ ions exchange with extracellular Na⁺ ions with resulting intracellular alkalisation [26, 27]. Fluorescence ratios were stored on a microcomputer for later analysis, after the calibration procedure (see below). To study the Na⁺/H⁺ exchange due to the antiport, the specific inhibitor EIPA (10⁻⁵ mol/l) was used. These same experiments of loading intracellular pH to 6 and studying external Na⁺ dependent H⁺ efflux (with and without EIPA) were also performed on cells treated with staurosporine (100 nmol/l).

To convert fluorescence ratios to pH units, a 15 point calibration curve was constructed by suspending cells in isotonic KCl buffer of the same composition used in loading intracellular pH to 6.0 (see

above). External pH was changed in the range 5.9 to 7.8 by addition of HCl. The fluorescence ratio is related to intracellular pH by the following equation [29]:

$$F = A + B(10^{pH - pK}) / (1 + 10^{pH - pK})$$

where F is the fluorescence ratio (500/439 nm), A and B are constants and pK the pK of intracellular dye. The parameters A, B and pK were determined for each individual by a non-linear least squares technique [30]. These constants were used to calculate internal pH values for the cells acid loaded to pH 6.0, before and during exposure to external Na⁺. The internal pH values were used to calculate the initial rate of change of pH after addition of the Na⁺, by calculating the regression coefficient for pH against time for the first 20 s of the record using a linear least squares technique. Buffering power was determined in cells with internal pH clamped at 6.0 using (NH₄)₂SO₄ as described above. The product of buffering power (units in mmol·l⁻¹·pH⁻¹) and rate of change of internal pH (units in pH units/min) gives external Na⁺ dependent H⁺ efflux (units in mmol·l⁻¹·min⁻¹). The difference between the total and EIPA-sensitive H⁺ efflux is the EIPA-sensitive proton efflux rate, a measure of Na⁺/H⁺ antiport activity. All measurements were in duplicate and repeated in the presence of 100 nmol/l staurosporine.

Coefficients of variation for the same individual on different days were: 11.8% for intracellular pH (expressed as H⁺ ion concentration); 7.3% for external Na⁺ stimulated H⁺ efflux of cells acid loaded to internal pH 6.0.

Effect of dioctanoyl glycerol on leucocyte intracellular pH. Leucocytes were suspended in the same NaCl medium used for determining resting intracellular pH. After establishing an unstimulated intracellular pH recording, 10 μmol/l (final concentration) dioctanoyl glycerol was added. The dioctanoyl glycerol was dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide (0.1% volume/volume) had no effect on intracellular pH. To determine the change in intracellular pH attributable to the Na⁺/H⁺ antiport, the response of cells to dioctanoyl glycerol in the presence of EIPA (10⁻⁵ mol/l) was determined. Buffering power of the cells was measured with (NH₄)₂SO₄ as described above. These experiments with and without EIPA were done in the absence and presence of 100 nmol/l staurosporine, to examine the stimulation of the Na⁺/H⁺ antiport by the dioctanoyl glycerol. Staurosporine was added to cells 1 h before the internal pH response to dioctanoyl glycerol was studied.

Statistical analysis

Data were analysed on an Oxstat statistics package (Microsoft Corporation, Reading, UK), using the paired and unpaired Student's *t*-test as appropriate. Plasma insulin and urinary albumin/creatinine

Table 2. Leucocyte intracellular pH, buffering power and Na⁺/H⁺ antiport characteristics in the presence and absence of 100 nmol/l staurosporine of the subjects in the study. Means and SD are reported

	Type 1 (insulin-dependent) diabetic subjects		Normotensive control subjects
	Albuminuria	No albuminuria	
Resting leucocyte measurements			
Intracellular pH	7.59 ± 0.14 ^b	7.46 ± 0.09 ^d	7.46 ± 0.09
Intracellular pH (+ staurosporine)	7.61 ± 0.17 ^b	7.46 ± 0.07 ^d	7.44 ± 0.12
Buffering power (mmol · l ⁻¹ · pH ⁻¹)	10.7 ± 7.0 ^b	16.1 ± 4.1 ^d	16.2 ± 4.9
Buffering power (+ staurosporine) (mmol · l ⁻¹ · pH ⁻¹)	8.8 ± 5.7 ^c	13.5 ± 2.8 ^d	15.6 ± 5.1
Na ⁺ /H ⁺ antiport measurements at internal pH 6.0			
EIPA-sensitive H ⁺ efflux (mmol · l ⁻¹ · min ⁻¹)	73.8 ± 17.2 ^c	57.3 ± 11.6 ^d	54.5 ± 8.5
EIPA-sensitive H ⁺ efflux (+ staurosporine) (mmol · l ⁻¹ · min ⁻¹)	54.9 ± 17.9 ^f	50.0 ± 12.8 ^e	52.6 ± 10.4
Difference in EIPA-sensitive efflux from staurosporine (mmol · l ⁻¹ · min ⁻¹)	18.9 ± 14.4 ^c	7.3 ± 7.9 ^{a, d}	1.6 ± 5.3

^a $p < 0.05$; ^b $p < 0.01$; ^c $p < 0.001$ compared with normal control subjects; ^d $p < 0.01$ compared to diabetic subjects with albuminuria^e $p < 0.01$; ^f $p < 0.001$ compared to values without staurosporine

EIPA = ethyl-isopropyl amiloride

Fig. 1. Effect of 100 nmol/l staurosporine on the ethyl-isopropyl amiloride (EIPA)-sensitive H^+ efflux of leucocytes with internal pH altered to 6.0 in 19 Type 1 (insulin-dependent) diabetic patients with (left panel) and 15 Type 1 diabetic patients without nephropathy (centre panel), and 16 control subjects (right panel). Each point represents the mean of a duplicate H^+ efflux determination in cells with or without 1 h preincubation with staurosporine. The EIPA-sensitive H^+ efflux is the difference between total efflux, and EIPA-insensitive efflux, measured in the presence of 10^{-5} mol/l EIPA

Fig. 2. Effect of different concentrations of staurosporine (— control, 20 nmol/l, --- 50 nmol/l, -.- 100 nmol/l) on the ethyl-isopropyl amiloride (EIPA)-sensitive change in intracellular pH of leucocytes from a normal subject, stimulated by addition of $10 \mu\text{mol/l}$ dioctanoyl glycerol. Similar results were obtained from four other subjects

ratios were normalised by log transformation before comparisons were made and are reported as geometric mean (geometric mean $\pm$ 1 SD). All other data are reported as mean $\pm$ 1 SD. The p values are 2 tailed.

Results

Table 1 shows the clinical characteristics of the subjects studied. Subjects were well matched for age and body mass index, and Type 1 diabetic subjects had similar durations of diabetes and daily insulin dosages. The diabetic subjects had higher systolic blood pressures than the normal control subjects, although diastolic blood pressures were very similar. The glycated haemoglobin and fasting glucose levels in the diabetic subjects with and without raised urinary albumin/creatinine ratios were very similar and higher than those of normal subjects. Free insulin levels were similar in all groups.

Leucocytes isolated from diabetic subjects with raised albumin/creatinine ratios had a significantly raised intracellular pH (7.59 ± 0.14) compared to normal control subjects (7.46 ± 0.09 , $p < 0.002$) or diabetic subjects with normal albumin/creatinine ratios (7.46 ± 0.09 , $p < 0.003$; Table 2). These differences in resting intracellular pH persisted even after incubation in 100 nmol/l staurosporine for 1 h (Table 2). The buffering power of cells from diabetic subjects with albuminuria was lower than that of control or diabetic subjects without albuminuria.

To examine the activity of the Na^+/H^+ antiport, cells were clamped to an internal pH of 6.0 using the double ionophore technique described earlier. Leucocytes from Type 1 diabetic subjects with albuminuria had significantly higher EIPA-sensitive proton efflux rates than both normal and diabetic subjects without albuminuria (Table 2). Furthermore, incubation of leucocytes in 100 nmol/l staurosporine, a protein kinase C inhibitor, led to a very significant fall in EIPA-sensitive proton efflux rates in diabetic subjects with albuminuria (Table 2, Fig. 1, $p < 0.001$), with smaller changes in diabetic subjects without albuminuria (Table 2, Fig. 1, $p < 0.003$). The proton efflux rates in normal control subjects did not change significantly with staurosporine.

The effect of a protein kinase C stimulator dioctanoyl glycerol was determined in resting leucocytes. Dioctanoyl glycerol led to an initial fall in intracellular pH with a nadir at about 3 min, followed by a gradual rise towards the resting intracellular pH level reaching a plateau by 10 min. In the presence of EIPA (10^{-5} mol/l) the intracellular pH fell but failed to rise subsequently, indicating that this rise in intracellular pH was due to activation of the Na^+/H^+ antiport. The difference between these two records gave the EIPA-sensitive change in intracellular pH which is due to activation of the Na^+/H^+ antiport by dioctanoyl glycerol (Fig. 2). This activation was inhibited by increasing concentrations of staurosporine (Fig. 2), indicating that the effect of dioctanoyl glycerol was via stimulation of protein kinase C. Table 3 shows the effects

Table 3. Effect of 10 µmol/l dioctanoyl glycerol on intracellular pH with and without added ethyl-isopropyl amiloride (EIPA) (10⁻⁵ mol/l) and/or staurosporine (100 nmol/l). The change in intracellular pH is defined as the difference between initial pH and final pH plateau 10 min after addition of dioctanoyl glycerol in the absence (A) and the presence of EIPA (B). The EIPA-sensitive change in pH (C) is the difference between the pH changes in the absence (A) and presence (B) of EIPA. The change in H⁺ concentration (D) due to Na⁺/H⁺ antiport stimulation by dioctanoyl glycerol is the product of the EIPA-sensitive change in pH (C) and the buffering power. Means and 1 SD are reported

	Type 1 (insulin-dependent) diabetic subjects		Normotensive control subjects
	Albumin- uria	No albumin- uria	
No staurosporine			
(A) Change in pH	-0.04 ± 0.13	-0.03 ± 0.08	-0.07 ± 0.12
(B) Change in pH (+ EIPA)	-0.36 ± 0.16	-0.31 ± 0.10	-0.38 ± 0.11
(C) EIPA-sensitive change in pH	0.32 ± 0.15	0.28 ± 0.11	0.32 ± 0.08
(D) Change in [H ⁺] (mmol/l)	3.0 ± 2.2 ^b	4.4 ± 1.5	5.1 ± 2.0
Staurosporine 100 nmol/l added			
(A) Change in pH	-0.08 ± 0.05	-0.06 ± 0.04	-0.05 ± 0.03
(B) Change in pH (+ EIPA)	-0.12 ± 0.04	-0.13 ± 0.03	-0.11 ± 0.03
(C) EIPA-sensitive change in pH	0.04 ± 0.06	0.07 ± 0.05	0.06 ± 0.04
(D) Change in [H ⁺] (mmol/l)	0.4 ± 0.8 ^a	1.0 ± 0.8 ^c	1.0 ± 0.8

^a $p < 0.05$; ^b $p < 0.01$ compared to normal control subjects

^c $p < 0.05$ compared to diabetic subjects with albuminuria

of dioctanoyl glycerol on intracellular pH, where change in intracellular pH was the difference between initial and final pH values (10 min after addition of the diacyl glycerol). The change in intracellular pH in the presence and absence of EIPA was very similar in the three groups. The change in intracellular pH attributable to activation of the Na⁺/H⁺ antiport (defined as difference in the change in intracellular pH in the presence and absence of EIPA) was not different in the three groups. However, cells from Type 1 diabetic subjects with albuminuria had significantly lower buffering power than normal control subjects and diabetic subjects without albuminuria (Table 2). Thus, the change in intracellular H⁺ concentration due to activation of the Na⁺/H⁺ antiport by dioctanoyl glycerol was lower in Type 1 diabetic subjects with albuminuria (3.0 ± 2.2 mmol/l) than normal control subjects (5.1 ± 2.0 mmol/l, $p < 0.01$; Table 3). Diabetic subjects without albuminuria had similar EIPA-sensitive changes in H⁺ concentration after dioctanoyl glycerol (4.4 ± 1.5 mmol/l) as normal control subjects. Table 3 also shows the effect of staurosporine (100 nmol/l) on the changes in intracellular pH after dioctanoyl glycerol. The activation of the Na⁺/H⁺ antiport by the diacyl glycerol was largely abolished. However, the EIPA-sensitive change in intracellular H⁺ concentration was still signifi-

cantly lower in diabetic subjects with albuminuria (0.4 ± 0.8 mmol/l) than normal control subjects (1.0 ± 0.8 mmol/l, $p < 0.03$) or diabetic subjects without albuminuria (1.0 ± 0.8 mmol/l, $p < 0.03$). This may be because staurosporine at 100 nmol/l does not completely inhibit the activation of the Na⁺/H⁺ antiport by dioctanoyl glycerol (Fig. 2).

Discussion

We had previously reported that the leucocyte Na⁺/H⁺ antiport activity was raised in Type 1 diabetic subjects with raised albumin/creatinine ratios [19] leading to intracellular alkalinisation. This increased antiport activity may be an indication of increased growth factor activity [11, 19] or due to an inherited predisposition to hypertension, as suggested for very similar findings on the erythrocyte Li⁺/Na⁺ exchange in diabetic nephropathy [5, 6, 10]. In this present study, we have tested the hypothesis that protein kinase C plays an important role in activating the Na⁺/H⁺ antiport in diabetic subjects with albuminuria.

The protein kinase C inhibitor staurosporine had a very dramatic inhibitory effect on the EIPA-sensitive proton efflux rate of acid loaded leucocytes (internal pH of 6.0) in the diabetic subjects with increased albumin/creatinine ratios, restoring the increased rates of proton efflux to levels similar to that of normal subjects. In contrast, staurosporine had no effect on the leucocyte EIPA-sensitive proton efflux of normal subjects and a smaller effect on diabetic subjects without albuminuria. This indicates that protein kinase C may be more active in diabetic subjects with albuminuria than normal control subjects. However, although staurosporine is the best commercially available inhibitor of protein kinase C, it has inhibitory effects on other protein kinases [20]. An alternative interpretation of our results would be an increased activity of these other protein kinases in diabetic nephropathy. Furthermore, diabetic subjects without albuminuria may also have a lesser degree of activation of these kinases, including protein kinase C. This may be a direct effect of glucose itself, for it has been demonstrated that endothelial cells incubated in 22.2 mmol/l glucose have elevated membrane bound (and hence activated) protein kinase C, with a parallel decrease in cytosolic kinase C levels [31]. The diabetic subjects with albuminuria may have activated protein kinase levels (including protein kinase C) over and above the stimulatory effect of chronic hyperglycaemia itself. The reason for this is unclear, though it may be linked to the activation of protein kinase C demonstrated in hypertension [15] and the documented higher blood pressures of relatives of patients with diabetic nephropathy [6].

Leucocytes from diabetic subjects with raised albumin/creatinine ratios had lower buffering power than normal. The lower buffering power in this diabetic group is unlikely to be purely due to the higher intracellular pH per se, because we have shown that buffering power stays constant at intracellular pH greater than about 7.0, and

risks with intracellular acidification below 7.0 [26]. Cellular buffering power is dependent not only on cytosolic composition but also on uptake (or release) of H⁺ ions from organelles, e.g. the acid vesicle system. Whether ionic pumping abnormalities on the plasma membrane coexist with similar changes on membranes of intracellular organelles is unknown at present, but may account for differences in cellular buffering power.

Activation of protein kinase C by dioctanoyl glycerol led to similar EIPA-sensitive changes in intracellular pH in all groups of subjects studied. However, those diabetic subjects with albuminuria had significantly lower EIPA-sensitive changes in intracellular H⁺ ion concentration attributable to the stimulation of the Na⁺/H⁺ antiport. Staurosporine abolished most of the EIPA-sensitive change in H⁺ ion concentration due to the dioctanoyl glycerol. However, in the presence of staurosporine, diabetic subjects with albuminuria still had significantly lower changes in intracellular H⁺ concentration than normal. This may be because at the concentration of staurosporine used, the inhibition of protein kinase C is not complete. The reduced stimulatory effect of dioctanoyl glycerol on the Na⁺/H⁺ antiport of leucocytes from diabetic subjects with albuminuria could have two explanations. Firstly, the increased activity of membrane bound protein kinase C in the resting state may leave a smaller pool of cytosolic protein kinase C that can be activated by the added diacyl glycerol. Secondly, the higher resting activity of protein kinase C may mean that the Na⁺/H⁺ antiport is already substantially activated by phosphorylation [32, 33], and any further activation of kinase C by dioctanoyl glycerol may result in a smaller increase in antiport phosphorylation (and hence activation).

The lack of effect of staurosporine on the resting intracellular pH of cells from all the groups of subjects may not be surprising since intracellular H⁺ ions have a cooperative effect upon their own efflux [11]. The Hill coefficient of the antiport for internal H⁺ ions is about 2 [27] so that at physiological pH, the antiport is almost inactive [27]. In acid loaded cells, there is marked activation and the effects of kinase C inhibition may be more easily demonstrated.

In summary, Type 1 diabetic subjects with increased albumin/creatinine ratios have elevated leucocyte Na⁺/H⁺ antiport activities compared to normal control subjects and diabetic subjects without albuminuria. This elevated antiport activity is reversed by incubation with staurosporine, a protein kinase inhibitor. Thus protein kinase activity (including protein kinase C) in diabetic subjects with albuminuria may be higher in the resting state though hyperglycaemia itself could explain part of the activation. This higher endogenous activity led to the reduced antiport activation by exogenously added diacyl glycerol. As staurosporine is not completely specific for protein kinase C, other kinases may also be involved. The reasons for increased protein kinase activity are at present unknown though a predisposition to essential hypertension [15] or elevated phospholipase C activity [18] are possibilities that need to be explored.

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