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Protein kinase C effects on nerve function, perfusion, Na^{+},K^{+}-ATPase activity and glutathione content in diabetic rats

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Abstract

Aims//hypothesis. Increased protein kinase C activity has been linked to diabetic vascular complications in the retina and kidney, which were attenuated by protein kinase C antagonist treatment. Neuropathy has a vascular component, therefore, the aim was to assess whether treatment with WAY151 003 or chelerythrine, inhibitors of protein kinase C regulatory and catalytic domains respectively, could correct nerve blood flow, conduction velocity, Na^{+},K^{+}-ATPase, and glutathione deficits in diabetic rats.
Methods. Diabetes was induced by streptozotocin. Sciatic nerve conduction velocity was measured in vivo and sciatic endoneurial perfusion was monitored by microelectrode polarography and hydrogen clearance. Glutathione content and Na^{+},K^{+}-ATPase activity were measured in extracts from homogenised sciatic nerves.
Results. After 8 weeks of diabetes, sciatic blood flow was 50 % reduced. Two weeks of WAY151 003 (3 or 100 mg/kg) treatment completely corrected this deficit and chelerythrine dose-dependently improved nerve perfusion. The inhibitors dose-dependently corrected a 20 % diabetic motor conduction deficit, however, at high doses (> 3.0 mg/kg WAY151003; > 0.1 mg/kg chelerythrine) conduction velocity was reduced towards the diabetic level. Sciatic Na^{+},K^{+}-ATPase activity, 42 % reduced by diabetes, was partially corrected by low but not high dose WAY151 003. In contrast, only a very high dose of chelerythrine partially restored Na^{+},K^{+}-ATPase activity. A 30 % diabetic deficit in sciatic glutathione content was unchanged by protein kinase C inhibition. The benefits of WAY151 003 on blood flow and conduction velocity were blocked by nitric oxide synthase inhibitor co-treatment.
Conclusion/interpretation. Protein kinase C contributes to experimental diabetic neuropathy by a neurovascular mechanism rather than through Na^{+},K^{+}-ATPase defects. [Diabetologia (1999) 42: 1120–1130]

Keywords Neuropathy, nerve conduction, blood flow, protein kinase C, diacylglycerol, oxidative stress, diabetic rat.

Changes in protein kinase (PK) C activity have been implicated in the complications of diabetes mellitus. In retina, blood vessels, kidney and heart, PKC activity is increased [1], perhaps due to raised de novo synthesis of diacylglycerol (DAG). A recent study has shown that PKC inhibitor treatment prevented the development of impaired retinal blood flow, renal glomerular hyperfiltration and microalbuminuria in diabetic rats [2] and this is compatible with the notion that PKC activation contributes to the aetiology of retinopathy and nephropathy.

In another complication-prone tissue, peripheral nerve, PKC activity is reduced or unchanged [3–6]. Moreover, one neurochemical explanation of neuro-

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Abbreviations: DAG, Diacylglycerol; NCV, nerve conduction velocity; NOS, nitric oxide synthase; PK, protein kinase; GSH, reduced glutathione; GSSG, oxidised glutathione.

pathic changes involves diminished nerve phophoinositide turnover, leading to decreased DAG availability and reduced PKC activity. In turn, this could cause a Na^+,K^+-ATPase activity deficit, reduced nerve conduction velocity (NCV) and degenerative changes in axons and Schwann cells [7]. On that view, PKC inhibitors would be expected to exacerbate diabetic nerve dysfunction.

Also ischaemia contributes to diabetic neuropathy, as noted in studies on nerve blood flow and oxygen tension in patients and animal models [8–10] and from observations on the effects of peripheral vasodilators [10–12]. Increased vascular PKC activation causes increased contractile responses and diminished endothelium-dependent relaxation [13–15]. The latter is a characteristic of experimental diabetes and has been noted for vasa nervorum [16, 17]. In brain vessels, impaired vasodilation associated with diabetes and hyperglycaemia is reversed by PKC inhibition [18, 19]. Thus, from a vascular viewpoint, PKC inhibitors could improve vasa nervorum function, which would enhance perfusion and correct nerve dysfunction.

Oxidative stress also contributes to neurovascular dysfunction in experimental diabetes; nerve glutathione concentrations are reduced by diabetes and this may be corrected by antioxidant or aldose reductase inhibitor treatment [20, 21]. These agents also increase nerve blood flow [20, 22], however, it is not known whether this is responsible for the glutathione improvements.

The aim of this investigation was to further elucidate the potential role of PKC in diabetic nerve dysfunction and the relevance of neurochemical and vascular changes. To this end, we examined the effects of two drugs that differed in chemical structure and site of inhibition of PKC on sciatic NCV, endoneurial blood flow, Na^+,K^+-ATPase activity and glutathione content.

Materials and methods

Experimental groups and diabetes induction. Experiments were done in accordance with regulations specified in the United Kingdom "Animal Procedures Act, 1986" and the National Institutes of Health "Principles of Laboratory Animal Care, 1985 revised form." Two investigations were carried out in which diabetes duration was 8 weeks and drug treatment was given over the final 2 weeks. Diabetes was induced by intraperitoneal injection (40–45 mg/kg) of streptozotocin (Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK or Sigma, St Louis, Mo., USA) in mature 19-week-old male Sprague-Dawley rats obtained either from Aberdeen University breeding colony or from Charles River (Kingston, N. Y., USA). Investigations carried out in Aberdeen were concerned with nerve conduction and blood flow measurement whereas parallel studies in Princeton examined effects on sciatic nerve Na^+,K^+-ATPase activity and glutathione content.

Studies were carried out using two PKC inhibitors; 2,6-Diamino-*N*-([1-(1-oxotridecyl)-2-piperidinyl]methyl) hexanamide, WAY151 003 (Wyeth-Ayerst, Princeton, N.J., USA), which acts at the regulatory domain of the enzyme [23] and chelerythrine chloride (Alexis Corporation, San Diego, Calif., USA) which acts at the catalytic site [24]. Non-diabetic and 8 week diabetic control groups were used as well as treated groups. The first part of the WAY151 003 study was a dose-ranging experiment (0.1–100 mg/kg daily orally) on sciatic motor conduction velocity. Based on those results, two doses of WAY151 003 were selected for measurements of blood flow in separate groups of diabetic rats. Also included were groups of non-diabetic rats treated with a high dose of WAY151 003 and diabetic rats treated with an optimal dose of WAY151 003 in conjunction with a low dose (10 mg/kg daily) of the nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine. The purpose of the latter group was to provide further information on the extent to which the action of WAY151 003 on NCV depended on a vascular effect rather than a direct effect on nerve fibres or Schwann cells. While sciatic vasa nervorum endothelium contains NOS, the large myelinated fibres that dominate NCV measurements do not [25]. Previous studies have shown that the dose of N^G-nitro-L-arginine used has modest effects on NCV in non-diabetic rats while completely blocking the effects of agents such as aldose reductase inhibitors and antioxidants in diabetic rats [10, 26, 27]. A parallel set of groups was used for measurements of sciatic nerve Na^+,K^+-ATPase activity and glutathione content.

Chelerythrine chloride effects were studied with orally given daily doses of 0.01, 0.1 and 1.0 mg/kg for nerve function and blood flow in diabetic rats. An additional dose of 3.0 mg/kg was subsequently used for biochemical studies.

Sciatic nerve motor conduction velocity. Motor NCV was measured as described previously [28] in sciatic branches to tibialis anterior muscle, which is representative of the whole sciatic nerve in terms of susceptibility to diabetes and treatment effects. Briefly, under intraperitoneal urethane (1.0–1.5 g/kg) or thiobutabarbital (Zeneca; 50–100 mg/kg) anaesthesia, the trachea was cannulated for artificial ventilation and the sciatic nerve was exposed between sciatic notch and knee. Nerve temperature was monitored with a near-nerve probe and was kept at 36–37 °C using radiant heat. Core temperature was regulated between 36.5 and 37.5 °C.

Sciatic nerve blood flow. Sciatic endoneurial blood flow was measured by microelectrode polarography and H_2 clearance as previously described [27, 29]. Briefly, rats were anaesthetised by thiobutabarbital, the trachea was cannulated and a carotid cannula was used to monitor systemic blood pressure. Rats were given neuromuscular blockade using d-tubocurarine (Sigma, 2 mg/kg by way of the carotid cannula) and were artificially ventilated. The level of anaesthesia was monitored by observing any reaction of blood pressure to manipulation, and supplementary thiobutabarbital was given as necessary. Body core temperature was maintained at 37–38 °C and near-nerve temperature was maintained at 35–38 °C by radiant heat applied to a pre-warmed mineral oil pool that bathed the exposed sciatic nerve. A glass-insulated H_2-sensitive electrode was inserted into the middle of the nerve. We added 10 % H_2 to the inspired gas, the proportions of O_2 and N_2 being adjusted to 20 % and 70 % respectively. When the electrode current had stabilised, the H_2 supply was shut off and clearance monitored. This was repeated at another nerve site. Mono- or bi-exponential curves were fitted by regression (Prism, Graphpad, San Diego, Calif., USA). The slow exponent was taken for nutritive capillary flow [8]. Vascular conductance was calculated by dividing blood flow by mean arterial blood pressure. Endo-

neurial perfusion variables were averaged from the two H_2 clearance measurements.

Sciatic nerve Na^+,K^+-ATPase, glutathione, diacylglycerol and protein kinase C. Rats were killed by thiobutabarbital overdose. Sciatic nerves were rapidly removed from their vertebral exit to the common peroneal bifurcation, cleaned and placed on ice for Na^+,K^+-ATPase or protein kinase C activity measurements or frozen on dry ice and stored at –80 °C for up to 7 days for subsequent quantification of glutathione or DAG concentrations.

Nerve Na^+,K^+-ATPase activity was assayed at 340 nm by monitoring the disappearance of NADH in an enzymatic reaction that coupled Na^+,K^+-ATPase, pyruvate kinase and lactate dehydrogenase activities [30], using a membrane enriched fraction prepared from the sciatic nerve as described previously [31]. In brief, sciatic nerves were desheathed, immersed in 20 mmol · l^{-1} Tris-HCl, pH 7.5, containing 150 mmol/l NaCl, 20 mmol/l KCl, 3 mmol/l $MgCl_2$, 1 mmol/l EDTA (homogenising buffer) and 0.5 mol/l sucrose and homogenised using a ground glass homogeniser. Preliminary experiments showed that the recovery of Na^+,K^+-ATPase activity was independent of whether the nerves were disrupted with a ground glass homogeniser, or a Polytron blade homogeniser, in contrast to findings in an earlier study [32]. A discontinuous 3-step sucrose gradient was prepared with the homogenate sandwiched between 1.2 and 0.25 mol/l sucrose layers. Samples were centrifuged at 140 000 *g* for 60 min at 4 °C. The opaque layer was collected, resuspended in homogenising buffer with 0.25 mol/l sucrose, centrifuged at 140 000 *g* for 30 min at 4 °C, and then resuspended in homogenising buffer with 0.25 mol/l sucrose. Aliquots of the membrane enriched fraction (30 µl) were made up to 1 ml in a solution containing 3 mmol/l ATP, 1 mmol/l phosphoenolpyruvate, 0.3 mmol/l NADH, 18 U lactate dehydrogenase, and 18 U pyruvate kinase, prepared in 50 mmol/l imidazole buffer, pH 7.3, with 100 mmol/l NaCl, 10 mmol/l KCl, and 5 mmol/l $MgCl_2$. This reaction mixture was then stabilised at 37 °C for 16 min and the NADH disappearance was monitored for 15 min without and with 750 µmol/l ouabain. Preliminary experiments established that similar levels of inhibition were obtained for ouabain concentrations between 200 µmol/l and 5.0 mmol/l, therefore, an intermediate concentration of 750 µmol/l was selected for maximal inhibition. The reaction was linear for at least 45 min after the stabilisation period. ATPase activity was defined as the rate of NADH oxidation, expressed as the equimolar amount of ATP converted to ADP. Ouabain-sensitive Na^+,K^+-ATPase activity was the difference between the rate before and after ouabain addition and was normalised to protein content. Chelerythrine or WAY151 003 added to the enzyme reaction mixture in the concentration range 10^{-9} to 10^{-7} mol/l had no effect on Na^+,K^+-ATPase activity.

Concentrations of free reduced (GSH) and oxidised (GSSG) glutathione were quantified with an enzymatic recycling assay [33] in the distal portion of the sciatic nerve, about 1 cm proximal to the common peroneal bifurcation. In brief, frozen nerves were allowed to thaw in 200 µl of 5 % solution of sulphosalic acid. Sample were then desheathed, weighed, minced and re-immersed in 10 µl of sulphosalic acid per mg wet weight of sample. During subsequent processing the samples were maintained at 4 °C. Preparations were disrupted by sonication and after 20 min extraction, homogenates were centrifuged for 10 min at 20 000 *g* and the pellets discarded. Separate assays were used to measure free GSH and GSH + GSSG. The residual concentration of sulphosalic acid in the supernatant was diluted to 1.0 % for the GSH assay and 0.15 % for GSH + GSSG, with sodium phosphate buffer, pH 7.5.

Standards were prepared from stock solutions of GSH and GSSG. Aliquots of diluted supernatant fractions and standards were transferred to individual wells in a 96-well plate. The reaction was initiated with the addition of 100 µl of a reaction mixture yielding a final concentration of 0.2 mmol/l NADPH, 0.15 mmol/l 5,5 ′-dithiobis [2-nitrobenzoic acid], and 1 U of glutathione reductase in 100 mmol/l sodium phosphate buffer, pH 7.5, with 1 mmol/l EDTA. The reaction was monitored at 405 nm. To quantify tissue GSSG, GSH was removed by the addition of 2-vinylpyridine to form a final concentration of 0.35 mol/l. To optimise the activity of glutathione reductase, the pH of the samples was adjusted to 7.0 with triethanolamine.

The precision of the assays for GSSG and GSH, measured as the inter and intra-day coefficient of variation for concentrations of standards ranging from 4.7 to 300 pmol/sample, was 8.1 ± 6.7 % for GSSG and 2.0 ± 0.8 % for GSH. The assay accuracy, expressed as the percent of the nominal value, was 97.7 ± 0.3 % for GSSG and 93.5 ± 2.7 % for GSH. The lower limit of quantitation for pure standards was 4.7 pmol/sample. Storage of samples at –80 °C for 14 days had no effect on tissue GSSG or GSSG + GSH concentrations. GSSG measurements were unaffected by the addition of 150 pmol/sample of GSH or 300 pmol of cysteine.

Total DAG concentrations were measured in the sciatic nerve from non-diabetic control rats, diabetic rats, and diabetic rats treated with WAY151 003. DAG was measured with a DAG kinase kit (RPN200, Amersham, Arlington Heights, Ill., USA) and the tissues were prepared as described previously [34]. In brief, frozen sciatic nerves were homogenised in a methanol, water and chloroform (2:1:2) mixture. The chloroform phase was recovered after centrifugation at 20 000 *g* for 5 min, and the phosphorylation reaction was done according to the manufacturer's instructions. The radio-labelled phosphatidic acid was separated by column chromatography (Amprep, Amersham) and counted. Preliminary experiments on control and diabetic rats showed that there were no relevant changes in DAG content comparing measurements on nerves that were freshly harvested with those on nerves that had been stored at –80 °C for 7 days. Furthermore, DAG measurements were not affected by the anaesthetic (halothane, thiobutabarbital or CO_2 overdose) used to kill the rats before samples were taken.

PKC activity was measured in sciatic nerves and retina from non-diabetic control and diabetic rats. Freshly isolated tissues were homogenised in buffer A (20 mmol/l Tris.HCl, pH 7.5, with 2 mmol/l EDTA, 0.5 mmol/l EGTA, 2 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulphonyl fluoride, 25 µg/ml leupeptin, 2 µg/ml pepstatin A and 0.33 mol/l sucrose), followed by centrifugation at 100 000 *g* for 30 min at 4 °C. The supernatant was retained as the tissue cytosolic fraction. The pellet was washed with buffer B (buffer A without sucrose) and was then resuspended in buffer B with 0.1 % triton X-100. After a 45 min incubation at 4 °C, and centrifugation at 100 000 *g* for 30 min at 4 °C, the supernatant was retained as the tissue membrane fraction. PKC activity in cytosolic and membrane fractions was partially purified by loading the extracts on an ion exchange column and eluting with buffer B containing 200 mmol/l NaCl. PKC activity was measured in a reaction mixture containing 20 mmol/l Tris-HCl, pH 7.5, 1.2 mmol/l calcium acetate, 100 µmol/l ATP with [γ-^{32}P] (120 cpm/pmol), 90 µmol/l of a PKC-specific peptide substrate with or without 0.03 mg/ml of phosphatidylserine, and 2.4 mg/ml phorbol myristate acetate (Biotrak PKC Enzyme Assay System, Amersham). After a 3 min incubation at 30 °C, the reaction was terminated by the addition of 300 mmol/l orthophosphoric acid cooled to 4 °C and an aliquot of the mixture was spotted onto

phosphocellulose disks and allowed to dry. The disks were washed with 5 % acetic acid and the radiolabel was quantified by liquid scintillation spectrometry.

Statistical analysis. Data are expressed as means ± SEM. They were first subjected to Bartlett's test for homogeneity of variances and were given a log transformation if necessary. One-way ANOVA was followed by the Student-Newman-Keuls test to estimate the significance of differences for between-group comparisons. Where log transformation failed to equalise group variances (GSH, WAY151 003 study), non-parametric Kruskal-Wallis ANOVA was followed by Dunn's multiple comparison test.

Results

Body weights for diabetic groups were in the range 371 ± 10 g – 423 ± 7 g and were not affected by treatment with WAY151 003 or chelerythrine. This was about 24 % reduced compared with the non-diabetic control groups (506 ± 8 g – 532 ± 7 g). Non-diabetic rats treated with the highest dose (100 mg/kg) of WAY151 003 for the last 2 weeks had a diminished weight gain (463 ± 4 g). Plasma glucose was 31.3 ± 0.7 mmol/l to 35.3 ± 0.9 mmol/l with diabetes, irrespective of PKC inhibitor treatment, which was about 3.7-fold increased compared with the non-diabetic range (7.8 ± 0.6–10.2 ± 0.4 mmol/l).

Sciatic motor conduction velocity and blood flow. The WAY151 003 dose response curve for motor NCV (Fig. 1) had an "inverted-U" shape. A 19.9 ± 0.9 % deficit with untreated diabetes was significantly ($p < 0.001$) improved by doses above 0.1 mg/kg; the optimal dose was about 3 mg/kg, which corrected the deficit by 92.4 ± 5.0 %. The log ED_{50} for the low dose limb of the curve was –0.62 ± 0.05, corresponding to about 0.24 mg/kg. At 10 mg/kg the effect on NCV was significantly less pronounced ($p < 0.01$) than at 3 mg/kg and at 100 mg/kg NCV was at the diabetic control level. High-dose (100 mg/kg) WAY151 003 treatment did not alter NCV in non-diabetic rats. In diabetic rats treated with the optimal (3 mg/kg) WAY151 003 dose in conjunction with N^G-nitro-L-arginine, NCV was reduced ($p < 0.001$) to the diabetic control level.

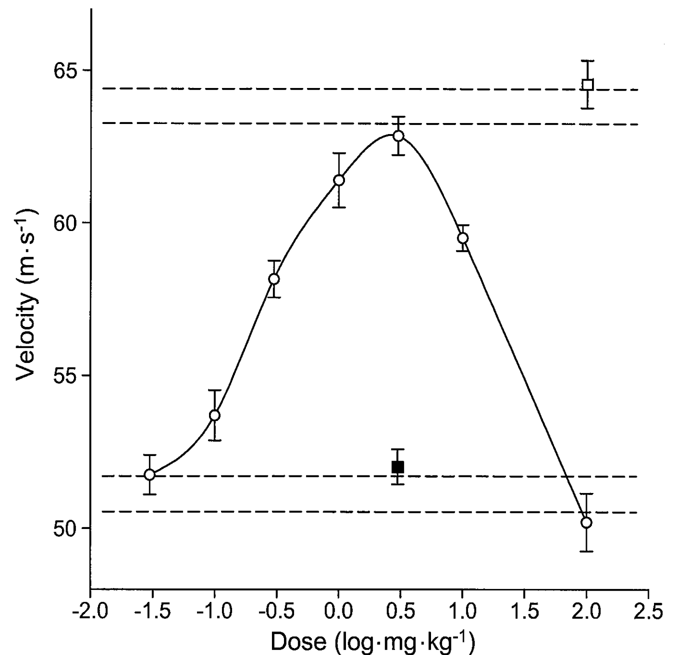

Fig. 1. Dose response relation for the effects of WAY151 003 on sciatic nerve motor conduction velocity. Diabetes duration was 8 weeks and treatment (0.03–100 mg · kg^{-1} · day^{-1}) was given for the last 2 weeks (○, $n = 6–10$). Also shown are data from a group of non-diabetic rats treated with 100 mg/kg day WAY151 003 for 2 weeks (□, $n = 10$) and a group of diabetic rats treated with an optimal dose of WAY151 003 (3 mg · kg^{-1} · day^{-1}) and co-treated with 10 mg · kg^{-1} · day^{-1} of the nitric oxide synthase inhibitor, N^G-nitro-L-arginine (■, $n = 10$). Data are group means ± SEM. The upper and lower pairs of dashed lines represent ± SEM for groups ($n = 10$) of non-diabetic and diabetic control rats respectively. The diabetic level was significantly ($p < 0.001$) exceeded for WAY151 003 doses in the range 0.3–10 mg/kg. At doses of 1 and 3 mg/kg, conduction velocity was not significantly different from that of the non-diabetic control group. At 10 mg/kg, conduction velocity had declined compared with 3 mg/kg ($p < 0.01$) and for 100 mg/kg, velocity was in the diabetic control range. Co-treatment with 10 mg/kg N^G-nitro-L-arginine attenuated the effect of 3 mg/kg WAY151 003 ($p < 0.001$) such that conduction velocity was in the diabetic control range

Sciatic nutritive endoneurial perfusion was examined in diabetic rats treated daily with WAY151003 at doses of 3 mg/kg and 100 mg/kg, which produced the two most extreme effects on NCV. A 50.4 ± 3.5 % reduction ($p < 0.001$) in blood flow (Fig. 2A) in the diabetic control group was completely corrected by the 3 mg/kg dose ($p < 0.001$) and 90.6 ± 12.0 % corrected ($p < 0.001$) by 100 mg/kg WAY151 003. In neither case was the result significantly different from that of the non-diabetic control group. When the 3 mg/kg dose was given with N^G-nitro-L-arginine co-treatment, blood flow was reduced ($p < 0.001$) to a level similar to that of the diabetic control group. Nutritive blood flow in non-diabetic rats was not significantly changed by 100 mg/kg WAY151 003.

Mean systemic blood pressure (Fig. 2B) varied between groups, with a tendency towards lowering with diabetes and WAY151 003 treatment, which was significant for both 3 mg/kg ($p < 0.001$) and 100 mg/kg ($p < 0.01$) doses. A trend towards relative hypotension in WAY151003-treated non-diabetic rats did not reach statistical significance. The highest blood pressure was noted for the WAY151 003 and N^G-nitro-L-arginine treated diabetic group, which was significantly raised ($p < 0.01$) compared with all other groups except the non-diabetic control rats, as

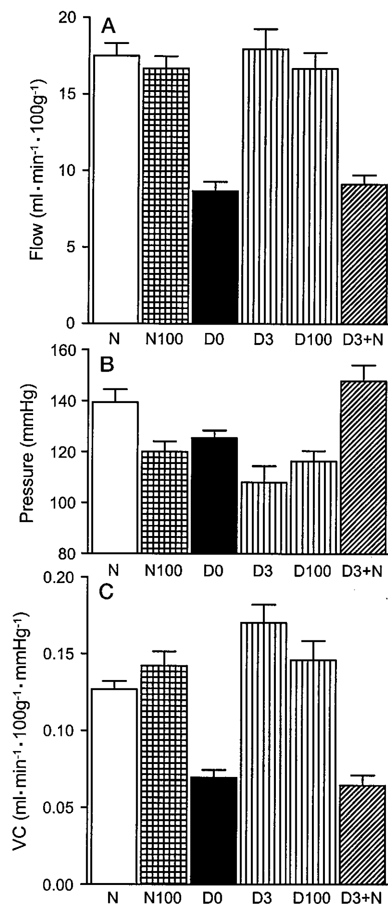

Fig. 2 A–C. Effects of diabetes and treatment on **A** sciatic nutritive endoneurial blood flow, **B** mean systemic blood pressure and **C** endoneurial vascular conductance (VC). Groups ($n = 10$); N, non-diabetic control; N100, non-diabetic treated daily for 2 weeks with 100 mg/kg WAY151003; D0, 8 week diabetic control; D3, D100, 8 week diabetic rats treated daily for the last 2 weeks with 3 mg/kg or 100 mg/kg WAY151 003 respectively; D3 + N, 8 week diabetic rats co-treated for the last 2 weeks with 3 mg/kg WAY151 003 and 10 mg/kg N^{G}-nitro-L-arginine. Data are mean + SEM. Statistics: flow; N, N100, D3 or D100 vs D0 or D3 + N, $p < 0.001$. Pressure; N vs N100, $p < 0.05$; N vs D3 or D100, $p < 0.01$; D3 + N vs D0, D3, D100, N100, $p < 0.01$. Vascular conductance, N, N100, D3 or D100 vs D0 or D3 + N, $p < 0.001$. All other comparisons NS

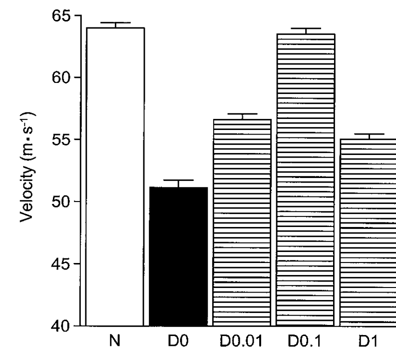

Fig. 3. Effects of chelerythrine on sciatic nerve motor conduction velocity. Groups ($n = 10$); N, non-diabetic control; D0, 8 week diabetic control; D0.01, D0.1 and D1; 8 week diabetic rats treated daily for the last 2 weeks with chelerythrine at doses of 0.01, 0.1 and 1 mg/kg respectively. Data are mean + SEM. Statistics, D0 vs N, D0.01, D0.1 or D1, $p < 0.001$; N vs D0.01 or D1, $p < 0.001$; D0.1 vs D0.01 or D1, $p < 0.001$; all other comparisons NS

expected from previous studies for that level of constitutive NOS inhibition [26, 27]. As vasa nervorum have poor pressure autoregulation [8], the perfusion data are also expressed as vascular conductance (Fig. 2C). This was 45.2 ± 3.9 % decreased by untreated diabetes ($p < 0.001$), which was completely corrected by both doses of WAY151003 ($p < 0.001$). A numerical trend towards supernormal conductance in treated diabetic and non-diabetic groups was not statistically significant. Conductance was reduced ($p < 0.001$) to the untreated diabetic level by N^{G}-nitro-L-arginine co-treatment.

NCV responses to chelerythrine chloride (Fig. 3) followed a broadly similar pattern to those for WAY151003. A 20.1 ± 0.9 % NCV reduction ($p < 0.001$) with diabetes was partially corrected ($p < 0.001$) by chelerythrine doses of 0.01 mg/kg (42.8 ± 3.4 %), 0.1 mg/kg (96.3 ± 3.7 %) and 1.0 mg/kg (30.6 ± 3.3 %). Both low and high doses were less effective ($p < 0.001$) than the intermediate dose.

The corresponding changes in nutritive endoneurial perfusion are shown in Fig. 4. Blood flow (Fig. 4A) was halved by diabetes ($p < 0.001$), and this was corrected ($p < 0.001$) by 51.0 ± 7.2 %, 92.4 ± 13.4 % and 101.7 ± 10.6 % by chelerythrine doses of 0.01, 0.1 and 1.0 mg/kg respectively. Mean systemic blood pressure (Fig. 4B) tended to be reduced in diabetic ($p < 0.05$) and chelerythrine treated diabetic rats compared with non-diabetic control rats at all doses ($p < 0.01$) and compared with the diabetic control group for the 1.0 mg/kg treatment group ($p < 0.05$). Vascular conductance (Fig. 4C) was reduced by diabetes to 55.4 ± 3.7 % ($p < 0.001$) of the value for the non-diabetic group. Conductance was increased ($p < 0.001$) to 90.9 ± 5.5 % of the non-diabetic value for 0.01 mg/kg chelerythrine treatment, and was supernormal by 23.1 ± 8.3 % ($p < 0.05$) for 0.1 mg/kg and 36.4 ± 10.3 % ($p < 0.01$) for 1.0 mg/kg groups.

Sciatic nerve biochemical measurements. Total sciatic nerve PKC activity did not differ between non-diabetic ($n = 9$) and diabetic ($n = 9$) rats, with 87.1 ± 10.1 and 82.8 ± 12.4 pmol of ^{32}P transferred $min^{-1} \cdot mg^{-1} \cdot protein^{-1}$, respectively. Similar percentages of total activity were associated with the membrane fraction in both groups (non-diabetic 40.6 ± 2.5 %; diabetic 46.9 ± 2.2 %). Total retinal PKC activity was also similar in non-diabetic and diabetic rats, 136.8 ± 12.7 and 106.2 ± 8.8 pmol of ^{32}P transferred $min^{-1} \cdot mg^{-1} \cdot protein^{-1}$, respectively. The level of ac-

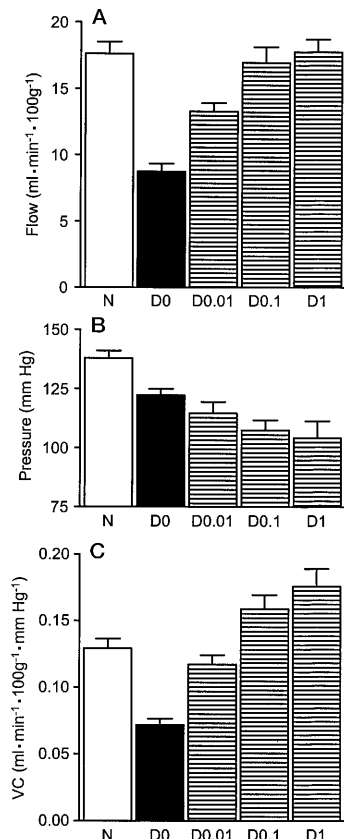

Fig. 4 A–C. Effects of chelerythrine on **A** sciatic endoneurial blood flow, **B** mean systemic blood pressure and **C** endoneurial vascular conductance (VC). Groups ($n = 8$–10); N, non-diabetic control; D0, 8 week diabetic control; D0.01, D0.1 and D1; 8 week diabetic rats treated daily for the last 2 weeks with chelerythrine at doses of 0.01, 0.1 and 1 mg/kg respectively. Data are mean + SEM. Statistics: flow; D0 vs N, D0.01, D0.1 or D1, $p < 0.001$; D0.01 vs N, D0.1 or D1, $p < 0.01$. Pressure; N vs D0, $p < 0.05$; N vs D0.01, $p < 0.01$; N vs D0.1 or D1, $p < 0.001$; D0 vs D1, $p < 0.05$. Conductance; D0 vs N, D0.01, D0.1 or D1, $p < 0.001$; N vs D0.1, $p < 0.05$; N vs D1, $p < 0.01$; D0.01 vs D0.1, $p < 0.01$; D0.01 vs D1, $p < 0.001$. All other comparisons NS

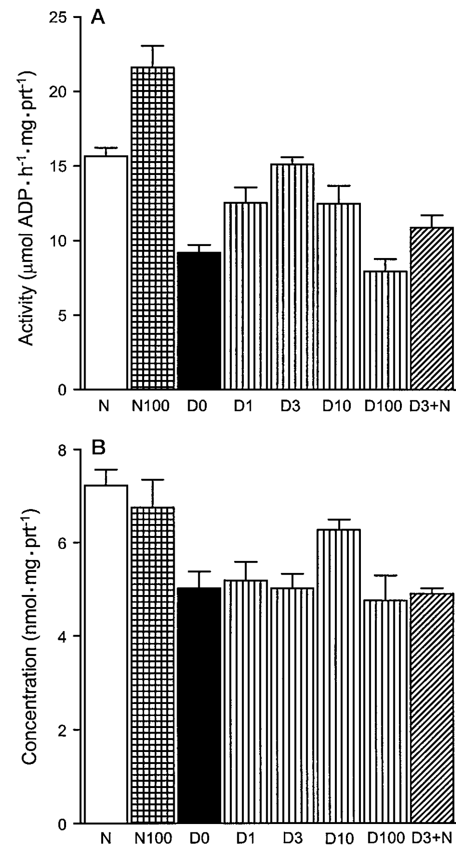

Fig. 5 A, B. Effects of WAY151 003 on sciatic nerve ouabain-sensitive Na^{+},K^{+}-ATPase activity **A** and reduced glutathione content **B**. Groups ($n = 7$–33); N, non-diabetic control; N100, non-diabetic treated daily for 2 weeks with 100 mg/kg WAY151003; D0, 8 week diabetic control; D1, D3, D10, D100, 8 week diabetic rats treated daily for the last 2 weeks with 1, 3, 10 or 100 mg/kg WAY151 003 respectively; D3 + N, 8 week diabetic rats co-treated for the last 2 weeks with 3 mg/kg WAY151 003 and 10 mg/kg N^{G}-nitro-L-arginine. Data are mean + SEM. Statistics: Na^{+},K^{+}-ATPase; N vs D0, D100 or D3 + N, $p < 0.001$; N vs D1 or D10, $p < 0.05$; D0 vs D1, $p < 0.05$; D0 vs D10, $p < 0.01$; D0 vs D3, $p < 0.001$; N100 vs all other groups, $p < 0.001$. Glutathione; N vs D0, D3 or D3 + N, $p < 0.001$; N vs D1 or D100, $p < 0.05$. All other comparisons NS

tivity associated with the retinal membrane fraction in diabetic rats (7.2 ± 0.7 %), however, was greater ($p < 0.01$) than that found in the non-diabetic control group (4.8 ± 0.5 %).

The sciatic nerve DAG content in non-diabetic rats (4.01 ± 0.22 $nmol^{-1} \cdot mg^{-1} \cdot protein^{-1}$; $n = 9$), was not different from that found with untreated diabetes (4.16 ± 0.40 $nmol^{-1} \cdot mg^{-1} \cdot protein^{-1}$; $n = 9$). DAG concentrations in nerves from diabetic groups ($n = 9$) treated daily with WAY151003 at doses of 3 or 100 mg/kg, 4.40 ± 0.51 and 3.73 ± 0.43 $nmol^{-1} \cdot mg^{-1} \cdot protein^{-1}$, respectively, did not differ significantly from those in non-diabetic and diabetic control animals.

Diabetes caused a 41.5 ± 3.5 % reduction in ouabain-sensitive Na^{+},K^{+}-ATPase activity (Fig. 5A). This was partially corrected by WAY151003 at lower doses; 51.5 ± 16.0 % for 1 mg/kg ($p < 0.05$) and 91.5 ± 7.5 % for 3 mg/kg ($p < 0.001$). At 10 mg/kg the degree of correction was 50.8 ± 18.6 % ($p < 0.01$) and for the 100 mg/kg groupNa^{+},K^{+}-ATPase activity was in the untreated diabetic range. In contrast, Na^{+},K^{+}-ATPase activity was 37.9 ± 9.2 % supernormal ($p < 0.001$) in non-diabetic rats treated with 100 mg/kg WAY151 003. For diabetic rats treated with 3 mg/kg WAY151 003 and co-treated with N^{G}-nitro-L-arginine, Na^{+},K^{+}-ATPase activity was not significantly different from that of the diabetic control group.

Sciatic nerve GSH content (Fig. 5B) was reduced by diabetes (30.5 ± 4.9 %; $p < 0.001$). In diabetic rats given WAY151 003 treatment at doses of 1–100 $mg \cdot kg^{-1} \cdot day^{-1}$, GSH remained reduced compared with

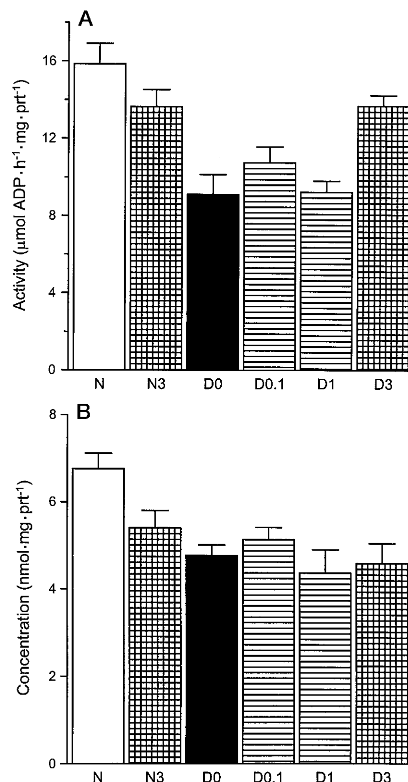

Fig. 6 A, B. Effects of chelerythrine on sciatic nerve ouabain-sensitive Na^+,K^+-ATPase activity **A** and reduced glutathione content **B**. Groups ($n = 12$–16); N, non-diabetic control; N3, non-diabetic rats treated daily for 2 weeks with 3 mg/kg chelerythrine; D0, 8 week diabetic control; D0.1, D1 and D3; 8 week diabetic rats treated daily for the last 2 weeks with chelerythrine at doses of 0.1, 1 and 3 mg/kg respectively. Data are mean + SEM. Statistics: Na^+,K^+-ATPase; N vs D0, D0.1 or D1, $p < 0.001$; D3 vs D0 or D1, $p < 0.01$; D3 vs D0.1, $p < 0.05$; N3 vs D0 or D1, $p < 0.01$; N3 vs D0.1, $p < 0.05$. Glutathione; N vs D0 or D0.1, $p < 0.01$; N vs D1 or D3, $p < 0.001$; N vs N3, $p < 0.05$. All other comparisons NS

the non-diabetic group ($p < 0.05$) although for the 10 mg/kg group, GSH content was not significantly different from non-diabetic or diabetic control groups. WAY151 003 treatment of non-diabetic rats or N^G-nitro-L-arginine co-treatment in diabetic rats did not affect GSH concentrations. Diabetes and PKC inhibitor treatment effects on total glutathione (GSH + GSSG, data not shown) approximated those for GSH.

The effects of chelerythrine on sciatic nerve ouabain-sensitive Na^+,K^+-ATPase (Fig. 6A) differed from those of WAY151 003. The deficit in untreated diabetes was 42.5 ± 6.4 % ($p < 0.001$). Daily chelerythrine doses of 0.1 and 1.0 mg/kg did not significantly change Na^+,K^+-ATPase compared with the untreated diabetic group, values remaining 32.2 ± 5.2 % and 41.8 ± 3.6 % lower ($p < 0.001$) respectively compared with the non-diabetic control group. A higher dose of chelerythrine (3.0 mg/kg) did not significantly change Na^+,K^+-ATPase activity in non-diabetic rats, however, in diabetic rats the deficit was corrected by 67.7 ± 8.3 % ($p < 0.01$) to give a value that was not significantly different from that of the non-diabetic control group. Sciatic nerve GSH content (Fig. 6B) was 29.5 ± 3.6 % reduced by diabetes ($p < 0.01$) and this was unaffected by 0.1–3.0 mg/kg chelerythrine treatment. In non-diabetic rats, however, 3.0 mg/kg chelerythrine reduced GSH content by 20.1 ± 5.9 % ($p < 0.05$).

Discussion

The data show that low-dose PKC inhibitor treatment corrects NCV deficits in diabetic rats. This probably reflects the action on vasa nervorum to increase nerve blood flow; vasodilators improve endoneurial perfusion and NCV in diabetic rats [9–12]. There are parallels with recent findings in the retina. Thus, retinal blood flow deficits were induced in non-diabetic rats when PKC was stimulated by phorbol esters and decreased blood flow in diabetic rats was corrected by PKC inhibition [1, 2].

Retinal changes in diabetes include increased DAG and PKC activation [1]; the latter was noted in this study as an increase in the activity in the membrane fraction. In contrast, there was no change in PKC activity or distribution for nerve, in agreement with a previous investigation [4] although the literature is inconsistent. Thus, one group found that diabetes reduced total cytosolic PKC activity while membrane activity and protein content were unchanged [3]. Another group analysed isoenzymes and found reduced cytosolic PKCα and βII [5,6]. Therefore, if there is a link between reduced PKC activity and nerve function or Na^+,K^+-ATPase activity, then the cytosolic PKC component should be involved either directly or indirectly. This is compatible with the lack of an acute PKC inhibitor effect on Na^+,K^+-ATPase activity in the membrane fraction of nerve homogenates. Diabetes did not change total nerve DAG, although a previous detailed analysis found a reduction in arachidonyl-containing species that are important PKC activators [35]. Some arachidonyl species, however, act as endogenous inhibitors; for example, DAGs containing 13-hydroxyoctadecadienoic acid inhibit PKCβ [36, 37], the elevation of which has been implicated in diabetic complications including neuropathy [2, 38]. Thus, the precise functional impact of reduced nerve arachidonyl DAGs in diabetes is uncertain.

NCV deficits in diabetic rats have been attributed to reduced Na^+,K^+-ATPase activity, consequent on impaired *myo*-inositol metabolism and diminished PKC activation which reduces the phosphorylation of the Na^+,K^+-ATPase α-subunit [39]. Na^+,K^+-ATPase activity may be corrected by aldose reductase inhibitors and L-carnitine derivatives [7, 40]. These

drugs also improve nerve perfusion in diabetic rats [22, 27, 29, 41]. Thus, although our data are contrary to a hypothesis linking reduced PKC activation to impaired nerve function, PKC inhibitor effects on Na^+,K^+-ATPase activity were examined to elucidate any linkages with NCV and blood flow. For WAY151003, Na^+,K^+-ATPase activity roughly paralleled the dose-dependent changes in NCV. In contrast, for chelerythrine only a very high dose, which would be expected to have deleterious effects on NCV, improved Na^+,K^+-ATPase activity. The reason for this between-drug difference is not clear but could reflect preferential inhibition of PKC isoforms. Thus, PKC inhibitor effects on NCV and Na^+,K^+-ATPase are dissociated. Vasodilator treatment with prazosin prevented sciatic NCV deficits although Na^+,K^+-ATPase activity remained at the diabetic level [42]. Evening primrose oil reduced Na^+,K^+-ATPase activity in diabetic rats, while preventing NCV deficits [43]. Furthermore, low-dose acetyl-L-carnitine corrected the diabetic Na^+,K^+-ATPase deficit while having no effect on NCV [40]. Together, this suggests that maximum ouabain-sensitive Na^+,K^+-ATPase activity in nerve homogenates correlates poorly with NCV. Possible reasons are that the Na^+,K^+-ATPase deficit is not large enough to affect NCV or that NCV is dominated by the largest myelinated fibres whereas all cell types contribute to Na^+,K^+-ATPase measures, perhaps masking specific large fibre changes. In non-diabetic rats, high PKC inhibitor doses had different effects on Na^+,K^+-ATPase activity, being supernormal with WAY151 003 but unchanged with chelerythrine. The former would not be expected to increase NCV, which is constrained by fibre diameter and myelination considerations. Thus, while recent data show that PKC effects on Na^+,K^+-ATPase are complex and in some tissues elevated PKC activity reduces Na^+,K^+-ATPase [44], overall there is no support for the view that PKC inhibitors improve NCV by their action on Na^+,K^+-ATPase.

The reduced NCV and Na^+,K^+-ATPase activity when diabetic rats were treated with an optimal WAY151 003 dose and co-treated with *N*^{G}-nitro-L-arginine suggests a complex relation between vascular and metabolic effects on nerve. Thus, the large myelinated fibres that dominate NCV do not contain NOS whereas the endothelium of vasa nervorum does [25]. This suggests that NCV effects of WAY151 003 are directly attributable to improved nerve perfusion. Any relation between Na^+,K^+-ATPase and nerve perfusion cannot be simple because improved perfusion with chelerythrine, or prazosin in another study [42], had no effect on Na^+,K^+-ATPase. Similarly, increased NCV and nerve perfusion with antioxidants, including vitamin E, trientine and *N*-acetyl-L-cysteine [45–47] were not paralleled by Na^+,K^+-ATPase improvements (T. C. Hohman, unpublished observations). In non-diabetic rats, chronic NOS inhibitor treatment caused parallel NCV and Na^+,K^+-ATPase reductions [26, 29, 48]. It is possible that the Na^+,K^+-ATPase deficit is an adaptive response to nerve hypoxia more related to energy conservation than to NCV. Thus, Na^+,K^+-ATPase activity accounts for about 70 % of resting nerve ATP consumption; a 40 % reduction with diabetes would limit the potential effects of reduced oxygen supply on other energy-requiring aspects of nerve function [49]. Coupled with increased anaerobic metabolism, this may explain why diabetic nerve ATP concentrations are normal while oxygen and biochemical measurements, such as mitochondrial NADH / NAD^+, indicate endoneurial ischaemia [8, 11, 22, 50].

High-dose WAY151 003 and chelerythrine treatment caused NCV to decline towards the diabetic level, although improved nerve perfusion was maintained. This dissociates NCV from blood flow and suggests that the microcirculatory benefits were opposed by direct deleterious actions on nerve cells. With WAY151003, this effect was unlikely to be due to a simple neurotoxicity because the same dose in non-diabetic rats did not affect NCV, and Na^+,K^+-ATPase activity was supernormal. It is possible that high-dose effects are apparent in diabetes because they are compounded by other aetiological factors not corrected by PKC inhibition, such as polyol pathway activity, advanced glycation, or impaired essential fatty acid metabolism. Alternatively, at high doses other kinases may be affected, although WAY151 003 is relatively specific for PKC and even at the highest dose PKA and Ca^{2+}/calmodulin-dependent protein kinase would be unaffected [23]. Chelerythrine is also relatively specific for PKC, with a median effective concentration 200-fold less than for PKA, tyrosine protein kinase and Ca^{2+}/calmodulin-dependent protein kinase [24]. WAY151 003 binds to the DAG regulatory site [23], and might be expected to inhibit all PKC isoforms that are DAG-activated, including α, β, γ, δ and ε. The isoform specificity of chelerythrine has not been reported. Peripheral nerve contains at least seven isoforms, βI, ε, βII, α, δ, γ and ζ [4–6]. The functional effects at different doses could, therefore, reflect dose-dependent inhibition of more than one isoform. WAY151 003 and chelerythrine act at different PKC sites, thus their NCV effects are likely to depend on PKC rather than another target enzyme.

The PKC system is associated with oxidative stress and is modulated by antioxidant action. PKC activity is raised by ischaemia or reperfusion and PKC inhibition protects against tissue damage [51]. In vascular tissues, glucose or phorbol ester stimulation of PKC increases free radical formation and impairs endothelium-dependent relaxation [15,19]. Vitamin E reduces PKC activity by stimulating DAG kinase activity and protects against oxidised LDL-mediated PKC increases in endothelial cells [1, 52]. Antioxidant effects

on nerve, however, are not mediated solely by PKC inhibition because they generally do not show the biphasic NCV dose-response relation found in this study [20, 45, 53–55].

A reduction in GSH content is an early indicator of oxidative stress in nerves of diabetic rats, which may be prevented or corrected by antioxidant and aldose reductase inhibitor treatments [20, 21]. In this study, nerve GSH and GSH plus GSSG were similarly decreased by diabetes, suggesting that the deficit depends mainly upon diminished glutathione synthesis rather than impaired recycling of GSSG to GSH. WAY151003 and chelerythrine had no effect on the GSH deficit, therefore, DAG and PKC inhibitors operate by a pathophysiological route differing at least partially from that of antioxidants or aldose reductase inhibitors. Moreover, while the latter drugs increase nerve perfusion [20, 22, 45–47, 53, 54], the PKC data suggest that this mechanism is not responsible for the GSH improvements.

PKC effects on vasa nervorum endothelial NOS could contribute to reduced blood flow in diabetes. Thus, PKC participates in the control of NOS gene expression and also phosphorylates NOS to reduce its activity; chelerythrine increases endothelial constitutive NOS mRNA and protein [56, 57]. Hyperglycaemia caused impaired endothelium-dependent relaxation, which was corrected by PKC inhibition [18, 19]. In non-diabetic rats, chelerythrine at a dose (10 nmol/l) comparable to that producing good NCV and blood flow effects, corrected impaired endothelium-dependent relaxation caused by 25 mmol/l glucose suffusion of cerebral arteries [19]. PKC activation also favours contraction of vascular smooth muscle by phosphorylation of contractile proteins and indirectly as part of the signalling cascade for the vasoconstrictors endothelin 1 and angiotensin II [17, 58, 59]. The renin-angiotensin and endothelin systems are upregulated in diabetic rats and vasa nervorum show enhanced vasoconstrictor responses [11, 60]. Thus, PKC activity in diabetes affects the vasodilation and vasoconstriction balance to strongly favour the latter, and this may explain the effects of PKC inhibitors on nerve blood flow and the susceptibility to low-dose N^{G}-nitro-L-arginine co-treatment.

In conclusion, PKC inhibition has profound effects on nerve function in diabetic rats. At low doses the beneficial effect on NCV stems mainly from increased nerve perfusion. At high doses, while improved perfusion is maintained, there appears to be a direct deleterious effect. The cause cannot be discerned from the data but could involve inhibition of multiple PKC isoforms. A β-specific PKC inhibitor has recently been developed that improves vascular function in retina and kidney of diabetic rats [2] and the first indications are that this extends to nerve tissue [38, 61]. Thus, the PKC inhibitor treatment approach may be broadly applicable to diabetic complications, which should be examined in clinical trials.

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