Reduced $\text{Na}^+ + \text{K}^+$ -ATPase activity in peripheral nerve of streptozotocin-diabetic rats: a role for protein kinase C?

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Summary. Six weeks after induction of diabetes, the rate of ouabain-sensitive $^{86}\text{Rb}^+$ accumulation, a parameter which reflects $\text{Na}^+ + \text{K}^+$ -ATPase pumping activity, was significantly reduced in endoneurial preparations of sciatic nerve from untreated diabetic rats compared with that in control rats (Trial 1, 0.19 ± 0.09 versus 0.48 ± 0.13 pmol/min per mg wet weight of tissue, $p < 0.001$; Trial 2, 0.27 ± 0.16 versus 0.47 ± 0.18 , $p < 0.01$). This decrease in ouabain-sensitive $^{86}\text{Rb}^+$ uptake was not observed in nerves from diabetic rats maintained on sorbinil (an aldose reductase inhibitor) or *myo*-inositol diets. Protein kinase C activity was demonstrated in the soluble fraction of a sciatic nerve homogenate

by assaying for lipid-activated, Ca^{2+} -dependent phosphorylation of calf thymus histone. No significant difference in the time course of kinase C activity was observed between cytosol fractions of nerve homogenates from control and diabetic rats (control, 6.22 ± 0.97 pmol ^{32}P incorporated/mg cytosol protein in 50 min; diabetic, 5.32 ± 0.71). Three low molecular weight neural proteins (each with $M_r < 29,000$) were identified as substrates for protein kinase C.

Key words: Streptozotocin diabetes, diabetic neuropathy, *myo*-inositol, aldose reductase inhibitor, $\text{Na}^+ + \text{K}^+$ -ATPase, protein kinase C.

Experimental diabetes in the rat is associated with a decrease in motor nerve conduction velocity [1]. Carbohydrate metabolism in peripheral nerve is independent of insulin control and therefore, glucose levels in nerve reflect the blood glucose concentration [2]. Following induction of diabetes, the glucose concentration is markedly elevated in rat sciatic nerve. Glucose is metabolised via the polyol pathway resulting in an accumulation of sorbitol and fructose. These changes are accompanied by a decrease in the free *myo*-inositol concentration [3, 4]. It has been proposed that the conduction defect in diabetic nerve may occur as a result of a relationship between the increased polyol pathway activity, the decreased *myo*-inositol concentration and an alteration in the activity of the enzyme $\text{Na}^+ + \text{K}^+$ -ATPase [5].

Initial experiments in this study were designed to investigate reports [6, 7] that $\text{Na}^+ + \text{K}^+$ -ATPase activity is lower in sciatic nerve homogenates from diabetic rats compared with that in homogenates from control rats. In Trial 1, rates of $^{86}\text{Rb}^+$ uptake by endoneurial preparations of sciatic nerve were compared in diabetic and age-matched control rats. $^{86}\text{Rb}^+$ ions are tracers for K^+ ions and the rate of ouabain-sensitive $^{86}\text{Rb}^+$ uptake by endoneurial preparations provides a direct

measure of $\text{Na}^+ + \text{K}^+$ -ATPase pumping activity in the tissue. In Trial 2, rates of $^{86}\text{Rb}^+$ uptake were compared in untreated diabetic rats, diabetic rats maintained on diets containing either the aldose reductase inhibitor, sorbinil (CP-45634 (+)-6-fluoro-spiro [chroman-4,4'-imidazolidine]-2',5'-dione, Pfizer, Sandwich, Kent, UK), or *myo*-inositol and age matched control rats.

The major function of *myo*-inositol is as a precursor for the synthesis of inositol-containing phospholipids, the hydrolysis of which releases inositol-1,4,5-trisphosphate and diacylglycerol, second messengers which mobilise intracellular Ca^{2+} stores and activate protein kinase C respectively [8]. Some preliminary evidence for a link between phosphoinositide metabolism and $\text{Na}^+ + \text{K}^+$ -ATPase activity has been reported. In peripheral nerve from alloxan-diabetic rabbits, protein kinase C activators were found to normalise ouabain-inhibitable oxygen uptake, a parameter taken to reflect $\text{Na}^+ + \text{K}^+$ -ATPase activity [9].

In the present study, protein kinase C activity in sciatic nerves from control and diabetic rats was measured directly by assaying for lipid-activated, Ca^{2+} -dependent protein phosphorylation. Endogenous neural substrates for kinase C were also investigated.

Materials and methods

Animal preparation

Diabetes was induced in male Wistar rats by a single i.p. injection of streptozotocin (Sigma, Poole, Dorset, UK) at a dose of 60 mg/kg body weight in 0.2 ml of 50 mmol/l sodium citrate buffer (pH 4.5). Age- and weight-matched control rats received a similar volume of buffer alone. Animals whose blood glucose concentration exceeded 16 mmol/l two days after injection were included as diabetic in the studies. In Trial 2, the treated diabetic rats were maintained on diets supplemented with sorbinil (400 mg/kg) or *myo*-inositol (Sigma) (10 g/kg). The weight of diet ingested per rat per day was monitored throughout the trial and the daily doses of sorbinil and *myo*-inositol were approximately 40 mg/kg body weight and 1 g/kg body weight respectively. Animals were killed 6 weeks after injection.

Measurement of $^{86}\text{Rb}^+$ uptake

Sciatic nerves were removed from the rats under sodium pentobarbitone anaesthesia ('Sagatal', May & Baker, Dagenham, Essex, UK) (60 mg/kg body weight by i.p. injection). Two ligatures (with 5/0 grade silk) were applied to each nerve before excision, one at the sciatic notch and the other approximately 4 cm distal to the first. The epineurium and the perineurial membrane were removed from the ligated nerves using the method of Gillon & Hawthorne [10] and two more ligatures were applied at the centre of the resulting endoneurial preparation. The nerve was bisected between these ligatures to yield two endoneurial preparations from each sciatic nerve. Endoneurial preparations were incubated at 37°C in Krebs-Henseleit bicarbonate ringer containing 4% (w/v) bovine serum albumin, 5 mmol/l glucose and 0.5 mmol/l *myo*-inositol with a gas phase of O_2/CO_2 (19:1, v/v) and in the presence of 20 $\mu\text{mol/l}$ $^{86}\text{RbCl}$ (1.8 mCi/mg Rb, Amersham International, Amersham, Bucks., UK). The proximal endoneurial preparation from each sciatic nerve was incubated in the presence of 1 mmol/l ouabain (Sigma). $^{86}\text{Rb}^+$ uptake was terminated by washing the endoneurial preparations twice in ice-cold 0.1 mol/l MgCl_2 (pH 7.0). The tissue was homogenised in 1.5 ml of distilled H_2O :0.17 mol/l ZnSO_4 :0.15 mol/l $\text{Ba}(\text{OH})_2$ (13:1:1, by volume), centrifuged at 600 g for 5 min and the supernatant taken for measurement of Cerenkov radiation. Ouabain-sensitive $^{86}\text{Rb}^+$ uptake was calculated by subtracting $^{86}\text{Rb}^+$ uptake by the proximal endoneurial preparation in the presence of ouabain from $^{86}\text{Rb}^+$ uptake by the distal endoneurial preparation in the absence of the inhibitor.

Sugar and sugar alcohol determination

The epineurium was removed from segments of sciatic nerve which were then weighed and homogenised in 1.5 ml of distilled H_2O :0.17 mol/l ZnSO_4 :0.15 mol/l $\text{Ba}(\text{OH})_2$ (13:1:1, by volume). Following centrifugation, the supernatants were lyophilised with 50 μg of α -methyl-D-mannoside (Sigma) as an internal standard. Free sugars and sugar alcohols in the lyophilised extracts were converted to their trimethylsilyl ether derivatives [11] by reaction with 200 μl of pyridine/hexamethyldisilazane/trimethylchlorosilane (10:2:1, by volume) for 15 min at room temperature. Samples were analysed using the procedure previously described [12].

Estimation of protein kinase C

Kinase activity was assayed by following the phosphorylation of an exogenous substrate, calf thymus histone. The protocol used was based on that of Kitano et al. [13] with some modification. Sciatic nerves from 4 rats were desheathed (final weight approximately 150 mg) and homogenised in 8 ml of 20 mmol/l Tris-HCl (pH 7.5) containing 0.25 mol/l sucrose, 2 mmol/l EDTA, 10 mmol/l ethyleneglycol-bis-(β -aminoethyl ether)N,N'-tetraacetic acid (EGTA)

and 1 mmol/l phenylmethylsulphonyl fluoride. The homogenate was centrifuged for 60 min at 100,000 g to give soluble (supernatant) and particulate (pellet resuspended in homogenisation buffer) fractions. Kinase activity was assayed in a reaction mixture (250 μl) containing 20 mmol/l Tris-HCl (pH 7.5), 10 mmol/l MgCl_2 , 500 $\mu\text{mol/l}$ ATP (Sigma), 1 $\mu\text{mol/l}$ [γ - ^{32}P]-ATP (0.5–3.0 Ci/mmol, Amersham), 83 $\mu\text{g/ml}$ histone IIAS (Sigma), 1 mmol/l dithiothreitol, 200 $\mu\text{mol/l}$ CaCl_2 , 2 μg of phosphatidylserine (Sigma), 0.7 μg of diolein (Sigma) and enzyme extract (15–20 μg protein). Phosphatidylserine and diolein were added to the mixture as lipid micelles. The reaction was started by the addition of [γ - ^{32}P]-ATP. Basal activity was measured in the presence of 0.5 mmol/l EGTA instead of CaCl_2 , phosphatidylserine and diolein. After incubation at 30°C, the reaction was terminated by the addition of 1 ml of 6.25% (w/v) trichloroacetic acid (TCA) in 10 mmol/l phosphoric acid and 200 μl of 6.25 mg/ml bovine serum albumin. The acid-precipitable material was washed by dissolving in 1 ml of 0.1 mol/l NaOH and re-precipitating with 1 ml of 10% (w/v) TCA before washing twice with 2 ml of 5% (w/v) TCA. The final TCA precipitates were dissolved in 0.6 ml of 100% formic acid and then added to 4 ml of scintillant for counting. Lipid-activated, Ca^{2+} -dependent protein kinase C activity (expressed in pmol ^{32}P transferred from ATP to histone per mg cytosol/particulate fraction protein) was calculated to be the difference between the rate of phosphorylation in the presence of Ca^{2+} , phosphatidylserine and diolein and that in the presence of Ca^{2+} alone.

Phosphorylation and separation of neural proteins

To investigate the phosphorylation of endogenous proteins, the cytosol fraction from a sciatic nerve homogenate was incubated for 50 min under the conditions described for protein kinase C estimation, but in the absence of histone. In order to obtain enough protein to form a TCA precipitate without the use of bovine serum albumin as a carrier, the incubation volumes were increased 10-fold. Samples were electrophoresed in one dimension in sodium dodecyl sulphate (SDS)/polyacrylamide gels by a modification of the method of Laemmli [14]. The separation gel contained a gradient of 5–15% (w/v) acrylamide, 0.375 mol/l Tris-HCl (pH 8.8) and 0.1% (w/v) SDS. The stacking gel contained 4% acrylamide, 0.135 mol/l Tris-HCl (pH 6.8) and 0.1% SDS. The electrophoresis buffer was 25 mmol/l Tris/0.192 mol/l glycine buffer, containing 0.1% SDS. The electrophoresis was conducted at a constant current of 30 mA. After staining with Coomassie Brilliant Blue, the gels were dried and subjected to fluorography with Fuji RX X-ray film.

M_r values of the phosphoproteins were estimated using the following proteins, with the indicated M_r values, as standards: myosin, 205,000; β -galactosidase, 116,000; phosphorylase B, 97,400; bovine serum albumin, 66,000; ovalbumin, 45,000; and carbonic anhydrase, 29,000 (Sigma).

Statistical analysis

Results are expressed as means $\pm$ SD. The significance of difference between the means was calculated using an unpaired Student's t-test. A statistically significant difference was accepted for $p < 0.05$.

Results

Animals

During the course of the studies (Table 1), the control rats increased in weight whereas the untreated diabetic rats lost, on average, 26% (Trial 1) and 28% (Trial 2) of their initial weights over the six week period. Diabetic animals maintained on sorbinil and *myo*-inositol diets

Table 1. Animal weights, serum glucose and nerve sugar and sugar alcohol concentrations of control and streptozotocin-diabetic rats six weeks after injection

	Initial weight (g)	Final weight (g)	Serum glucose (μmol/l)	Free sugar and sugar alcohol concentrations in sciatic nerve (μmol/g wet weight)		
				Glucose	Fructose	Myo-Inositol
<i>Trial 1</i>						
Control	245 ± 18 (19)	323 ± 25 (19)	7.7 ± 0.7 (19)	5.36 ± 1.08 (8)	0.87 ± 0.25 (8)	3.33 ± 0.75 (8)
Diabetic	250 ± 16 (15)	184 ± 31 ^a (15)	42.8 ± 8.6 ^a (15)	10.89 ± 0.69 ^a (6)	5.84 ± 0.95 ^a (6)	2.07 ± 0.20 ^a (6)
<i>Trial 2</i>						
Control	304 ± 10 (14)	370 ± 21 (14)	8.0 ± 1.2 (14)	4.73 ± 0.84 (4)	1.10 ± 0.59 (4)	3.20 ± 0.31 (4)
Untreated Diabetic	282 ± 6 (13)	203 ± 24 ^a (13)	46.9 ± 4.4 ^a (13)	10.79 ± 0.96 ^a (4)	5.63 ± 0.84 ^a (4)	2.02 ± 0.18 ^a (4)
Diabetic + Sorbinil	293 ± 10 (13)	242 ± 30 ^b (13)	44.5 ± 4.3 ^a (13)	10.42 ± 1.58 ^a (4)	0.91 ± 0.17 (4)	2.84 ± 0.15 (4)
Diabetic + Inositol	295 ± 7 (13)	239 ± 30 ^b (13)	48.4 ± 10.2 ^a (13)	10.63 ± 0.86 ^a (4)	5.45 ± 1.08 ^a (4)	2.80 ± 0.34 (4)

Results are expressed as means $\pm$ SD for the number of animals given in parentheses.

^a $p < 0.001$ versus age-matched control rats; ^b $p < 0.01$ versus untreated diabetic rats

Fig. 1. Time course of $^{86}\text{Rb}^+$ uptake by endoneurial preparations from control rat sciatic nerve. Each point represents the mean $\pm$ SD of at least 4 separate determinations. Filled squares ■, total uptake; filled diamonds ◆, ouabain-insensitive uptake; open squares □, the difference between these two, representing ouabain-sensitive uptake

Fig. 2. Time course of ^{32}P incorporation into histone catalysed by kinases in soluble and particulate fractions from control rat sciatic nerve homogenate. Each point represents the mean $\pm$ SD of 4 separate experiments each conducted in triplicate. ■, phosphorylation in the presence of Ca^{2+} , phosphatidylserine and diolein, cytosol; ▨, phosphorylation in the presence of Ca^{2+} alone, cytosol; □, phosphorylation in the presence of Ca^{2+} , phosphatidylserine, and diolein, particulate fraction; ▤, phosphorylation in the presence of Ca^{2+} alone, particulate fraction

lost 17% and 19% of their initial weights over the same period of time. The serum glucose concentration was markedly elevated in all diabetic groups. Sciatic nerves from untreated diabetic rats had increased concentrations of free glucose and fructose, but had decreased concentrations of free *myo*-inositol compared with nerves from control rats. Administration of sorbinil prevented the increase in fructose and decrease in *myo*-inositol observed in untreated diabetic rats. *Myo*-inositol supplementation had no effect on the fructose levels, but prevented the decrease in nerve *myo*-inositol levels. Diabetic rats used in the protein phosphorylation experiments lost on average 23% of their initial weights during the 6 week period after injection of streptozotocin. Two days after injection, each rat had a blood glucose concentration greater than 16 mmol/l (results not shown).

$^{86}\text{Rb}^+$ uptake studies

The time course of $^{86}\text{Rb}^+$ uptake by endoneurial preparations from control rat sciatic nerve is shown in Figure 1. The ouabain-insensitive uptake equilibrated more rapidly with the external $^{86}\text{Rb}^+$ concentration than did the ouabain-sensitive component. The ouabain-sensitive $^{86}\text{Rb}^+$ uptake increased as a percentage of total uptake with time. In subsequent experiments, endoneurial preparations were incubated for 20 min in the presence of $^{86}\text{RbCl}$.

Six weeks after induction of diabetes, the rate of total $^{86}\text{Rb}^+$ uptake was significantly diminished ($p < 0.001$ in Trial 1; $p < 0.01$ in Trial 2) in nerves from untreated diabetic rats compared with that in nerves from control rats (Table 2). This decrease was observed to occur wholly as a result of a reduction in the ouabain-

Table 2. ⁸⁶Rb⁺ uptake (in pmol/min per mg wet weight of tissue) by endoneurial preparations of sciatic nerve from diabetic and age-matched control rats

	Total uptake	Ouabain-insensitive uptake	Ouabain-sensitive uptake
<i>Trial 1</i>			
Control (20)	1.11 ± 0.19	0.63 ± 0.14	0.48 ± 0.13
Diabetic (17)	0.86 ± 0.17 ^a	0.67 ± 0.13	0.19 ± 0.09 ^a
<i>Trial 2</i>			
Control (19)	1.13 ± 0.20	0.65 ± 0.09	0.47 ± 0.18
Untreated Diabetic (16)	0.92 ± 0.15 ^b	0.65 ± 0.08	0.27 ± 0.16 ^b
Diabetic + Sorbinil (17)	1.28 ± 0.21	0.74 ± 0.13	0.54 ± 0.24 ^c
Diabetic + Inositol (17)	1.27 ± 0.23	0.74 ± 0.12	0.52 ± 0.23 ^c

Results are expressed as means ± SD for the numbers of sciatic nerves given in parentheses. ^a *p* < 0.001 versus age-matched control rats; ^b *p* < 0.01 versus age-matched control rats; ^c *p* < 0.001 versus untreated diabetic rats

Fig. 3. Time course of protein kinase C activity in soluble fractions from control (filled bars, ■) and diabetic (hatched bars, ▨) rat sciatic nerve homogenates. Each value represents the mean ± SD of either 4 (control) or 3 (diabetic) separate experiments each conducted in triplicate

sensitive component of the ⁸⁶Rb⁺ accumulation. No decrease in total or ouabain-sensitive ⁸⁶Rb⁺ was observed in nerves from diabetic animals treated with either sorbinil or *myo*-inositol. Ouabain-sensitive ⁸⁶Rb⁺ uptake in endoneurial preparations from diabetic rats maintained on sorbinil or *myo*-inositol diets was significantly higher than that in preparations from untreated diabetic rats (*p* < 0.001 in each case).

Estimation of protein kinase C

Protein kinase C activity was assayed in soluble and particulate fractions from control rat sciatic nerve homogenates with Ca²⁺ alone and in the presence of

Ca²⁺, phosphatidylserine and diolein (Fig. 2). Both Ca²⁺-activated kinase activity and total kinase activity were observed to be higher in the cytosol (7.66 ± 0.83 and 13.88 ± 0.32 pmol · mg protein⁻¹ · 50 min⁻¹ respectively) than in the particulate fraction (1.02 ± 0.15 and 1.05 ± 0.14 pmol · mg protein⁻¹ · 50 min⁻¹ respectively). The addition of phosphatidylserine and diolein to the cytosol fraction resulted in a 2-fold increase (*p* < 0.001 at each time point) in histone phosphorylation over that seen in the presence of Ca²⁺ alone. This increase indicates a stimulation of lipid-activated, Ca²⁺-dependent protein kinase C. No significant difference in the time course of lipid-activated, Ca²⁺-dependent histone phosphorylation was observed between control and diabetic soluble fractions (Fig. 3). The distribution of Ca²⁺-dependent and total kinase activities between soluble and particulate fractions of diabetic nerve was similar to that seen in control preparations (results not shown).

Neural substrates for protein kinase C

Kinase C activity in rat sciatic nerve was initially demonstrated by the phosphorylation of an exogenous substrate, calf thymus histone (Fig. 2). Evidence for lipid-activated, Ca²⁺-dependent phosphorylation of endogenous sciatic nerve proteins is shown in Figure 4. Incubation of control and diabetic sciatic nerve cytosol fractions with [γ-³²P]-ATP under basal conditions resulted in the incorporation of label into a number of neural proteins (mainly with M_r values in the range 66,000–45,000). Addition of Ca²⁺ did not enhance the labelling of these proteins. However, the addition of Ca²⁺ plus phosphatidylserine and diolein markedly stimulated ³²P incorporation into 3 low molecular weight bands (each with an M_r value < 29,000) in both control and diabetic fractions. Addition of particulate fraction proteins to the incubation did not result in the phosphorylation of any additional proteins under any of the above conditions (results not shown).

Discussion

In this study, the use of ⁸⁶Rb⁺ as a K⁺ tracer facilitated the measurement of ion fluxes across the intact membranes of respiring cells. Endoneurial preparations were employed to give a composite picture of the metabolism of nerve axons and Schwann cells in the absence of the heterogeneous population of epineurial cells. Ouabain-sensitive ⁸⁶Rb⁺ uptake was found to be inhibited in endoneurial preparations of sciatic nerve from diabetic rats. This demonstration of diminished Na⁺ + K⁺-ATPase activity in diabetic peripheral nerve corroborates the reports of previous studies in which Na⁺ + K⁺-ATPase activity was assayed in homogenates of sciatic nerve by measuring the release of either inorganic phosphate [6] or ADP [7]. Inhibition of the

Fig. 4. **a** Coomassie Blue stain of control (C) and diabetic (D) rat sciatic nerve cytosol proteins separated by sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis. **b** Autoradiograph of the above gel. The data shown is representative of 3 separate experiments

Na^+/K^+ pump occurred concomitantly with the changes in nerve sugar metabolism characteristic of hyperglycaemia, i.e. increased flux through the polyol pathway and a decrease in the concentration of free *myo*-inositol. This alteration in sugar metabolism and the decrease in conduction velocity which occurs in peripheral nerve in streptozotocin-diabetic rats are unrelated to the potential side-effects of streptozotocin and can be prevented by administration of insulin [3, 15]. If the inhibition of $\text{Na}^+ + \text{K}^+$ -ATPase activity were a consequence of streptozotocin toxicity rather than altered sugar metabolism, it is unlikely that the decrease in nerve conduction velocity would be reversed by treatment with insulin. Moreover, in this study, the reduction in ouabain-sensitive $^{86}\text{Rb}^+$ uptake seen in nerves from untreated rats after 6 weeks of diabetes was not observed in either sorbinil or *myo*-inositol treated diabetic animals. There is, therefore, no reason to suppose that the reduction in $\text{Na}^+ + \text{K}^+$ -ATPase activity is related to streptozotocin toxicity. The results presented here suggest that neural $\text{Na}^+ + \text{K}^+$ -ATPase activity is influenced primarily by the concentration of *myo*-inositol rather than polyol pathway activity, since inhibition of the enzyme is prevented by *myo*-inositol supplementation even though levels of fructose remain high. This conclusion is in accord with previously published data from experiments with sciatic nerve homogenates [7, 16].

Evidence for a relationship between *myo*-inositol and $\text{Na}^+ + \text{K}^+$ -ATPase activity was first presented by Charalampous [17], who showed that the enzyme was inhibited in KB cells grown in the absence of *myo*-inositol. Several lines of investigation have since strengthened the idea that *myo*-inositol and phosphoinositide metabolism may be linked to the operation of the Na^+/K^+ pump. Studies with rabbit kidney microsomes have indicated that phosphatidylinositol is required for the maintenance of $\text{Na}^+ + \text{K}^+$ -ATPase activity [18] and that the enzyme and its lipid activator exist in close association in the membrane [19]. It has been suggested that basal phosphoinositide turnover may control a major fraction of resting $\text{Na}^+ + \text{K}^+$ -ATPase activity in peripheral nerve [20, 21] and also in aorta [22]. The perturbation of such a regulatory system in response to a decrease in nerve *myo*-inositol concentration may be implicated in the reduced $\text{Na}^+ + \text{K}^+$ -ATPase pumping activity, the nerve conduction defect and the development of diabetic neuropathy. Reports that $\text{Na}^+ + \text{K}^+$ -ATPase activity in mouse embryo fibroblasts [23] and ouabain-sensitive oxygen uptake in diabetic peripheral nerve [9] are stimulated by phorbol esters have raised the intriguing possibility that protein kinase C may be implicated in the regulation of the Na^+/K^+ pump.

In the present study, neural kinase C activity was greater in the soluble than in the particulate fraction.

This finding fits with the hypothesis that in vivo the enzyme resides in the cytosol until it is activated and translocated to the plasma membrane [24, 25]. Cytosolic preparations of sciatic nerves from control and diabetic rats showed similar time courses of lipid-activated, Ca^{2+} -dependent histone phosphorylation. Thus, in vitro, kinase C enzymes from both control and diabetic nerves were stimulated to a similar degree by exogenous activators to phosphorylate an artificial substrate. However, it is possible that in vivo kinase C-dependent protein phosphorylation may differ in control and diabetic nerve. In the intact cell, diabetes may induce conditions which alter the release of diacylglycerol and hence the activity of the kinase or it may modulate the availability of substrates for the enzyme. It is interesting to note here that in Figure 4b, although identical amounts of protein were applied to each lane, the autoradiograph suggests that the bands representing proteins phosphorylated by kinase C are less heavily labelled in the diabetic sample than are those in the control. This may reflect lower concentrations of these substrates in the cytosol from diabetic nerve.

Three soluble endogenous neural substrates, each with an M_r value $< 29,000$, were observed for kinase C. This result implies that $\text{Na}^+ + \text{K}^+$ -ATPase, an integral membrane protein which comprises two different polypeptides, α (M_r 85,000–95,000) and β (M_r 40,000–60,000) [26], is not itself a substrate for the kinase. Although high concentrations of EDTA and EGTA, such as were present in the kinase assay, are inhibitory to many phosphatases [27], it is conceivable that residual phosphatase activity may have prevented the detection of all the potential substrates of kinase C. The putative relationship between kinase C and $\text{Na}^+ + \text{K}^+$ -ATPase remains a matter for investigation. It is possible that kinase C exerts an influence on the activity of the Na^+/K^+ pump by phosphorylating an intermediary regulatory protein. However, no evidence for such a mechanism exists.

The three proteins phosphorylated by kinase C in this study have yet to be identified, though it is possible that they may be incipient components of the myelin sheath. The major protein in peripheral nerve myelin is the P_0 glycoprotein which has an M_r value of $\sim 28,000$ [28]. Rodent sciatic nerve myelin also contains basic proteins with M_r values of 21,500, 18,500, 17,000 and 14,000 [29]. At least two of the myelin basic proteins in the central nervous system are known to be substrates for kinase C [30] and it is plausible that those in the peripheral nervous system are analogous in this respect. Once inserted into the myelin sheath, the P_0 glycoprotein and the basic proteins are intimately associated with the membrane [31]. However, newly formed P_0 , which is detectable in the cytoplasm by immunostaining [32], and basic proteins, which are synthesised on free ribosomes [33], might be expected to be present in the soluble fraction and so be accessible to activated cytosolic kinase C. In considering these

speculative identities for the substrates of kinase C, it is interesting to recall that the concentrations of the phosphoproteins are apparently diminished in cytosol from diabetic nerve. Demyelination is known to be associated with the development of diabetic neuropathy [34, 35] and there is evidence that the synthesis of myelin proteins is inhibited in experimental diabetes [36].

In conclusion, in peripheral nerve from diabetic rats, the observed decrease in Na^+/K^+ pumping activity seems to occur, at least in part, as a result of a reduction in the concentration of free *myo*-inositol. Although a low concentration of intracellular *myo*-inositol may inhibit the activity of kinase C in vivo, this effect is unlikely to be directly related to the inhibition of the Na^+/K^+ pump since none of the three substrates detected for the kinase was identified as a subunit of $\text{Na}^+ + \text{K}^+$ -ATPase. The mechanisms by which *myo*-inositol metabolism, kinase C-dependent protein phosphorylation and $\text{Na}^+ + \text{K}^+$ -ATPase activity are interrelated in the pathogenesis of diabetic neuropathy are alluring goals for further research.

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